Statistical procedures for calculating the residual virulence of *Brucella abortus* strain 19 (S19) and *Brucella melitensis* strain Rev 1 vaccines in mice: theoretical basis and practical applications

R. Pouillot [1], M.J. Grilló [2], J.L. Alabart [2], B. Garin-Bastuji [3] &
J.M. Blasco [2, 4]

(1) Agence Française de Sécurité Sanitaire des Aliments, Unité d'Appui Epidémiologique à l'Analyse de Risque, B.P. 19, 94701 Maisons Alfort Cedex, France
(2) Unidad de Sanidad Animal, Servicio Investigación Agroalimentaria, Diputación General de Aragón, Apartado 727, 50080 Zaragoza, Spain
(3) Agence Française de Sécurité Sanitaire des Aliments, Unité des Zoonoses Bactériennes, B.P. 19, 94701 Maisons Alfort Cedex, France
(4) Corresponding author

Summary
Regular control of the biological quality of live *Brucella abortus* strain 19 (S19) and *B. melitensis* strain Rev 1 vaccines is essential for the successful management of ruminant brucellosis in affected countries. The reference procedures recommended by the OIE (World organisation for animal health) and the European Pharmacopoeia include the determination of residual virulence, expressed as the recovery time 50 (RT_{50}), of the tested (problem) vaccine in a reference mouse model compared with the RT_{50} of the corresponding reference strains in the same assay. The underlying statistical procedure applied is based on a parallel line assay and a classical probit model. In practice, the currently recommended procedure for calculating the RT_{50} is based on a graphical method which has never been described in detail. This paper provides a full description of this graphical method with the aim of making the technique comprehensible and accessible to all interested biologists. The procedure is somewhat cumbersome and very few laboratories apply the OIE and European Pharmacopoeia recommendations on a regular basis. Moreover, since this reference graphical method shows some statistical inconsistencies, a dedicated internet interface has been developed to perform RT_{50} calculations and is now available free of charge on the web (www.afssa.fr/interne/rev2.html).

Keywords
Brucella live vaccines – Generalised linear model – Quality control – Recovery time 50 – Reed-Münch method – Residual virulence.

Introduction

Brucellosis is a widespread zoonotic disease, transmitted mainly from ruminants to humans. The only practical way to control the disease in endemic situations is by vaccination of small ruminants and cattle with the classical smooth *Brucella melitensis* Rev 1 and *B. abortus* S19 live vaccines, respectively (1, 13). However, the use of good quality commercial batches of these live, attenuated vaccines is an essential prerequisite for the success of vaccination campaigns (1). Quality control of

these vaccines is generally based on the exclusive examination of classical microbiological criteria (i.e. counting, lack of contamination and lack of colonial dissociation). This procedure has, however, been proven to be inadequate due to the existence of vaccine batches with acceptable microbiological characteristics but showing deficient immunological behaviour (3, 5, 6, 8, 9, 14). A particular and specifically designed mouse model has been demonstrated to be a useful tool for controlling the biological activity of both S19 and Rev 1 anti-*Brucella* vaccines (3, 4, 5, 6, 7, 8, 9, 12, 14,

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20, 21, 22). Furthermore, the OIE (World organisation for animal health) (18) and the European Pharmacopoeia (19) recommend checking representative seed lot batches of Rev 1 and S19 vaccines in this reference mouse model. Briefly, the model comprises two complementary studies based on spleen infection assays, i.e. determination of residual virulence and immunogenicity. Adequate residual virulence for a given strain is determined by calculating the particular recovery time 50 (RT_{50}), i.e. the time in weeks in which half of the inoculated mice are estimated as fully recovered from vaccine infection in the spleen, and then, by statistically comparing this RT_{50} value with that obtained with the corresponding Rev 1 or S19 reference strains in the same experiment. The model used to determine the residual virulence of both Rev 1 and S19 vaccines was conceived by the French National Institute for Agricultural Research (INRA: Institut National de la Recherche Agronomique) in Nouzilly, France (3, 4, 5, 9, 21, 22), and is currently accepted by the OIE as the reference model (18). This reference statistical method for calculating the RT_{50} was developed by modifying the graphical method of Bonet-Maury *et al.* (2), which has never been described in detail (4). The practical application of the method has been limited and very few laboratories perform the model on a regular basis due to lack of information and the relative complexity of the technique. The aim of this article is as follows:

– to describe this currently recommended graphical statistical method in a step-by-step manner and to provide practical examples on how the model can be used with real data

– to describe Rev 2, a dedicated internet interface, as a more precise and simple statistical alternative, which is easily accessible to most biologists for controlling the biological quality of both S19 and Rev 1 vaccines.

Experimental design, notations and theoretical statistical model

Experimental design and notations

The experimental design for calculating the RT_{50} has been described in detail elsewhere (4, 14, 19). Briefly, 32 female CD1 mice aged five to six weeks are inoculated subcutaneously with 10^8 colony-forming units (CFU)/mouse of the vaccine to be tested. Another group of 32 animals has to be inoculated and treated similarly with the corresponding *B. abortus* S19 or *B. melitensis* Rev 1 reference strains (both available from the INRA Laboratoire de Pathologie Infectieuse et Immunologie, Nouzilly, France; a reference *B. melitensis* Rev 1 strain can be also obtained from the European Pharmacopoeia, Strasbourg). Groups of eight animals per vaccine tested and per corresponding reference strain are then killed by cervical dislocation at three, six, nine and twelve weeks after inoculation. The spleens of the animals are aseptically removed, individually homogenised with 1 ml of sterile buffered saline solution and the whole homogenate smeared onto suitable

culture plates which are incubated in air at 37°C for five to seven days. The number of mice considered as 'cured' (no colonies isolated from the corresponding spleen) or 'infected' (at least one CFU isolated), at each slaughter time point, is then recorded.

The data to be collected from these experiments are as follows:

– j = (*REF, TEST*), the index of the vaccine strain used. *REF* corresponds to the reference strain, and *TEST* to the problem strain tested

– i = (1, 2, 3, 4), the index of the slaughter time

– T_i = (3, 6, 9, 12), the corresponding slaughter time point in weeks post-inoculation

– $N_{i,j}$ = the number of mice inoculated with strain j and slaughtered at each time index i. Usually, $N_{i,j}$ = 8 but some mice may die before slaughter, resulting in figures lower than 8

– $C_{i,j}$ = the number of mice inoculated with strain j found to be 'cured' at slaughter time index i

– $I_{i,j}$ = the number of mice inoculated with strain j found to be 'infected' at slaughter time index i, i.e. $I_{i,j} = N_{i,j} - C_{i,j}$

– $P_{i,j}$ = the probability that a mouse inoculated with strain j and slaughtered at time index i be cured. A set of data is shown in Table I as an example. Note that the figures given in this example will also be cited throughout this paper.

Biological assumptions underlying the mouse model

Basic comprehension of the statistical basis of the method is essential to understand the main weak points and possibilities for improving the method. The statistical method for determining the RT_{50} of the S19 and Rev 1 live anti-*Brucella* vaccines currently recommended by both the OIE (18) and the European Pharmacopoeia (19) – as well as the alternative Rev 2 interface proposed – are based on the probit model theory (10, 11, 17), using the two following assumptions:

– each inoculated mouse, indexed n, is assumed to have an individual curing time T_n. A given mouse slaughtered at a time T will thus be observed as cured if $T \geq T_n$. On the contrary, if mouse n is slaughtered at a time $T < T_n$, the animal will be considered as infected

– even using a well-defined mouse strain and a normalised experiment, curing time T_n varies according to the mice. This variability corresponds to the sum of the well-known biological variability and of the experimental variability. In these methods, parameter T is assumed to be distributed in the mouse population according to a normal distribution $N(\mu, \sigma)$, where μ is the mean and σ is the standard deviation.

Using these assumptions, the probit transformation of $P_{i,j}$, that is $\Phi^{-1}(P_{i,j})$ where $\Phi^{-1}(P_{i,j})$ is the inverse of the standard normal [i.e. $N(0, 1)$] the cumulative density function in $P_{i,j}$, is linearly

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Table I
Data used to determine the recovery time 50 (RT_{50}) values for a reference (*REF*) and problem (*TEST*) vaccine. Calculations were performed using the Reed and Münch method (23), with Bartlett's cunning

j	i	$T_{i,j}$	$N_{i,j}$	$C_{i,j}$	$C^*_{i,j}$	$CA_{i,j}$	$I_{i,j}$	$IA_{i,j}$	$NA_{i,j}$	$P_{i,j}$
							Calculated data			
REF	1	3	8	0	0.25	0.25	8 − 0.25 = 7.75	10.25 + 7.75 = 18	0.25 + 18 = 18.25	0.25/18.25 = 1.4%
	2	6	8	2	2	0.25 + 2 = 2.25	8 − 2 = 6	4.25 + 6 = 10.25	2.25 + 10.25 = 12.50	2.25/12.5 = 18%
	3	9	8	4	4	2.25 + 4 = 6.25	8 − 4 = 4	0.25 + 4 = 4.25	6.25 + 4.25 = 10.50	6.25/10.5 = 59%
	4	12	8	8	7.75	6.25 + 7.75 = 14	8 − 7.75 = 0.25	0.25	14 + 0.25 = 14.25	14/14.25 = 98%
TEST	1	3	8	0	0.25	0.25	8 − 0.25 = 7.75	13 + 7.75 = 20.75	0.25 + 20.75 = 21	0.25/21 = 1.2%
	2	6	8	1	1	0.25 + 1 = 1.25	8 − 1 = 7	6 + 7 = 13	1.25 + 13 = 14.25	1.25/14.25 = 8.8%
	3	9	8	4	4	1.25 + 4 = 5.25	8 − 4 = 4	2 + 4 = 6	5.25 + 6 = 11.25	5.25/11.25 = 48%
	4	12	8	6	6	5.25 + 6 = 11.25	8 − 6 = 2	2	11.25 + 2 = 13.25	11.25/13.25 = 86%

* Observed data modified by Bartlett's cunning
– j = (*REF*, *TEST*), index of the vaccine strain used
– i = (1, 2, 3, 4), index of the slaughter time
– $T_{i,j}$ = (3, 6, 9, 12), corresponding slaughter time point in weeks post-inoculation of mice inoculated with strain j
– $N_{i,j}$ = number of mice inoculated with strain j and slaughtered at each time index i
– $C_{i,j}$ = number of mice inoculated with strain j found to be 'cured' at slaughter time index i
– $CA_{i,j}$ = number of accumulated cured mice at slaughter time index i for strain j
– $I_{i,j}$ = number of mice inoculated with strain j found to be 'infected' at slaughter time index i
– $IA_{i,j}$ = number of accumulated infected mice at slaughter time index i for strain j
– $NA_{i,j}$ = total number of accumulated mice at slaughter time index i for strain j
– $P_{i,j}$ = probability that a mouse inoculated with strain j and slaughtered at time index i be cured

linked to the slaughtering time point $T_{i,j}$, as illustrated in Figure 1. Mathematically, this means that $P_{i,j}$ could be modelled using the following equation:

$$\Phi^{-1}(P_{i,j}) = \alpha_j + \beta_j T_{i,j}$$

where α_j is the intercept and β_j is the slope of the regression line of $P_{i,j}$ versus $T_{i,j}$ in a probit scale.

Once α_j and β_j are correctly estimated from the data, the $RT50$ for strain j, i.e. the time at which 50% of the mice inoculated with strain j are cured, may be calculated according to:

Eq. 1: $RT_{50,j} = -\dfrac{\alpha_j}{\beta_j}$

Statistical comparison of the RT_{50} obtained with the problem vaccine and the corresponding reference vaccine are based on the statistical parallel line assay theory (10, 11). This theory uses the underlying assumption that the curing time T of two populations of mice inoculated with different vaccine strains lead to two normal distributions, having the same standard error (i.e. the mouse-to-mouse variability is not modified) but possibly, different mean values (i.e. depending on the behaviour of the vaccine strain) (10, 11). If this assumption is correct, two parallel lines in a probit scale are obtained, as illustrated in Figure 2. The parallel line assay, and therefore comparison of the RT_{50} is only valid when, in a probit scale, the hypothesis of parallelism for the probability of being cured obtained with the two vaccines is not rejected. Once this has been validated, the vaccine strains are considered as statistically comparable. The strains are said to be biologically equivalent when their RT_{50} values are statistically similar, i.e. when the following null hypothesis H_0: $RT_{50,ref} = RT_{50,test}$ (Eq. 2) is not rejected ($RT_{50,ref}$ is the RT_{50} evaluated for the reference strain and $RT_{50,test}$ is the RT_{50} evaluated for the tested – problem – strain).

Note that, according to Eq. 1 and if the parallelism of the response is not rejected, this H_0 hypothesis may be rewritten as:

Eq. 3: $H_0 : \rho = \dfrac{\alpha_{ref} - \alpha_{test}}{\beta} = 0$

where β is the estimate of the common slope of the response; ρ is often referred to as the 'relative potency' (10, 11).
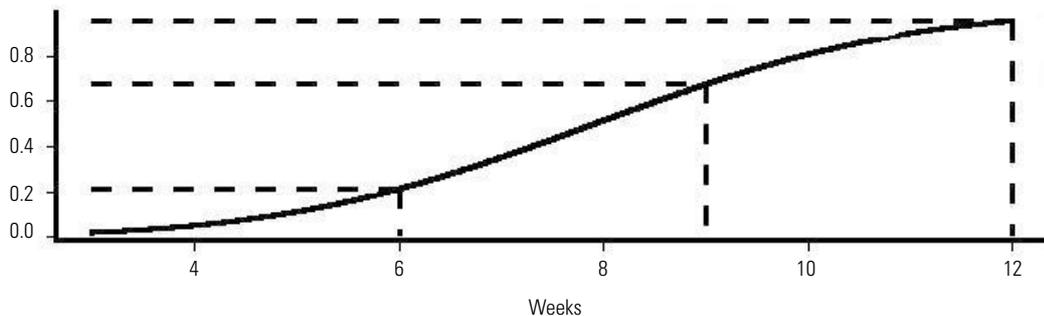
The graphical statistical method currently recommended by the World Organisation for Animal Health and the European Pharmacopoeia

The statistical method for calculating the RT_{50} of live anti-*Brucella* vaccines was developed by Dr N. Bosseray from INRA, by modifying the Bonet-Maury *et al.* method (2) used in the 1950s for calculating the lethal dose 50 (LD_{50}) of anti-typhoid vaccines. This method has been referred to, but never described in much detail (4). The following section provides a full

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a) The distribution of curing time in the mouse population follows a normal distribution
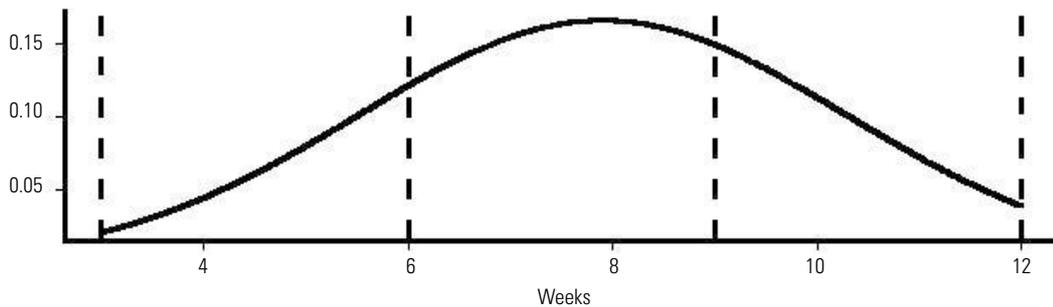
b) The probability for a mouse to be cured at a given time is the cumulative density function of the corresponding normal distribution

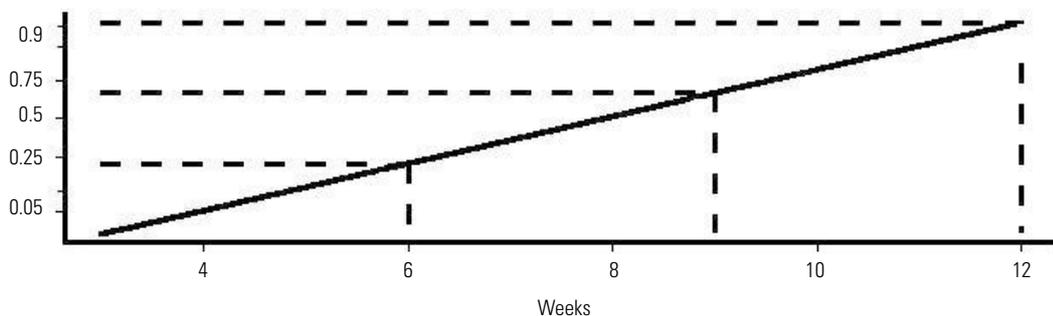c) Using a probit scale, this probability is a linear function of time

Fig. 1
The tolerance interpretation of the probit model for one strain

description of this graphical statistical method, as well as practical details for the application thereof.

The Bonet-Maury *et al.* method (2) is based on the Reed and Münch totals accumulated method (23), based on the intuitive suggestion that:

– one animal found cured at a given slaughter time point would also be found cured when slaughtered at subsequent time points

– one animal found infected at a given time point would also be found infected if slaughtered at previous time points.

For each slaughter time index *i*, all the animals found cured at the previous time points could then be accumulated.

The number of accumulated cured mice $CA_{i,j}$ at the slaughter time index *i* for strain *j* is calculated as follows:

$$CA_{i,j} = \sum_{k=l}^{i} C_{k,j}$$

and the number of accumulated infected mice $IA_{i,j}$ at slaughter time index *i* for strain *j* is calculated as follows:

$$IA_{i,j} = \sum_{k=i}^{4} l_{k,j}$$

Therefore, the total number of accumulated mice $NA_{i,j}$ at slaughter time index *i* for strain *j* is obtained as follows:

$$NA_{i,j} = CA_{i,j} + IA_{i,j}$$

The estimate of the percentage of cured accumulated mice $\tilde{P}_{i,j}$ at slaughter time index *i* for strain *j* is then obtained as follows:

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a) The distribution of curing time in the mouse population follows two normal distributions with (possibly) different means but equal standard errors
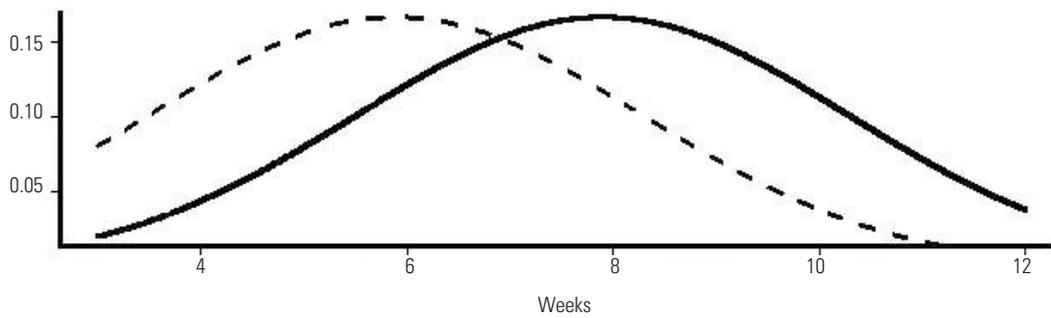
b) The probability for a mouse to be cured at a given time is thus the cumulative density function of the corresponding normal distributions
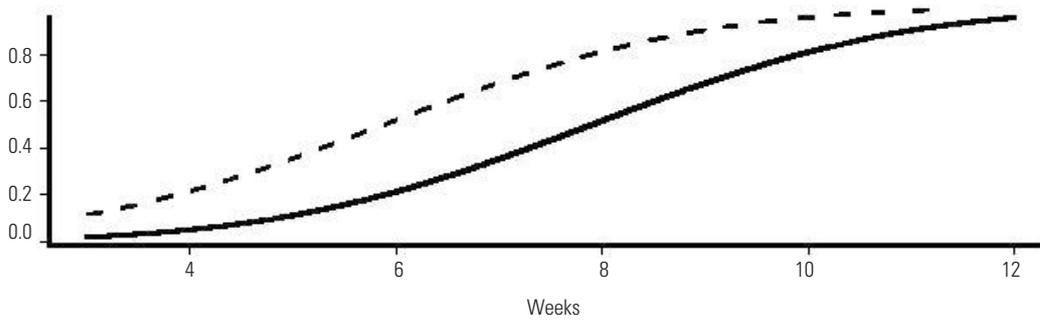
c) Using a probit scale, these probabilities draw two parallel lines
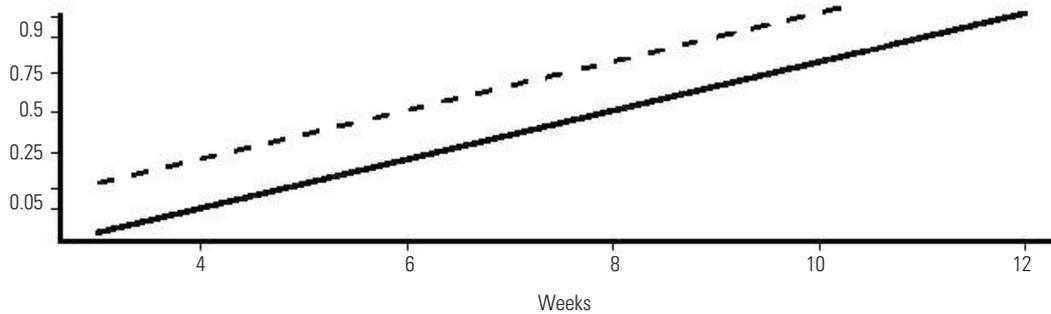
Fig. 2
The interpretation of the probit model using two vaccine strains

$$\tilde{P}_{i,j} = \frac{CA_{i,j}}{NA_{i,j}}.$$

Assuming the previously exposed hypothesis underlying the curing process of mice, the percentage of cured mice ($\tilde{P}_{i,j}$) is modelled according to the probit model. The graphical method proposed by the authors consists in obtaining a hand-plotted graphic representation on special probit paper (Fig. 3) of the estimated percentage of cured accumulated mice $\tilde{P}_{i,j}$ on the y-axis (on a probit scale) while time, $T_{i,j}$, is plotted on the x-axis (the RT_{50} graphical method includes manual logarithmic to arithmetic modification of the x-axis scale on Bonet-Maury *et al.* log-probit paper [2], to draw the regression line [4]). Since the probit transformation cannot be applied to 0% or 100% values, the use of Bartlett's cunning is recommended (2), with values of 0.25 and 7.75 being used when none or all mice are found cured, respectively. For the experiment to be valid, the percentages of cured accumulated mice should be included in the range from 16% to 84%. An example of data used to calculate the percentage of cured accumulated mice by means of the Reed and Münch method, including Bartlett's cunning, is shown in Table I.

Once the estimations are plotted on the modified probit paper, the technician should subjectively adjust, using a transparent ruler, the line that best fits with all observations. For hand-plotting the most probable regression line, the points corresponding to an estimated percentage of cured accumulated mice in the 5%-95% interval should have a higher weight than others. The determination of the RT_{50} values must be then performed graphically over the regression line by the point on the x-axis (weeks) corresponding to a probit value of 0.50 (50% of cured mice) on the y-axis.
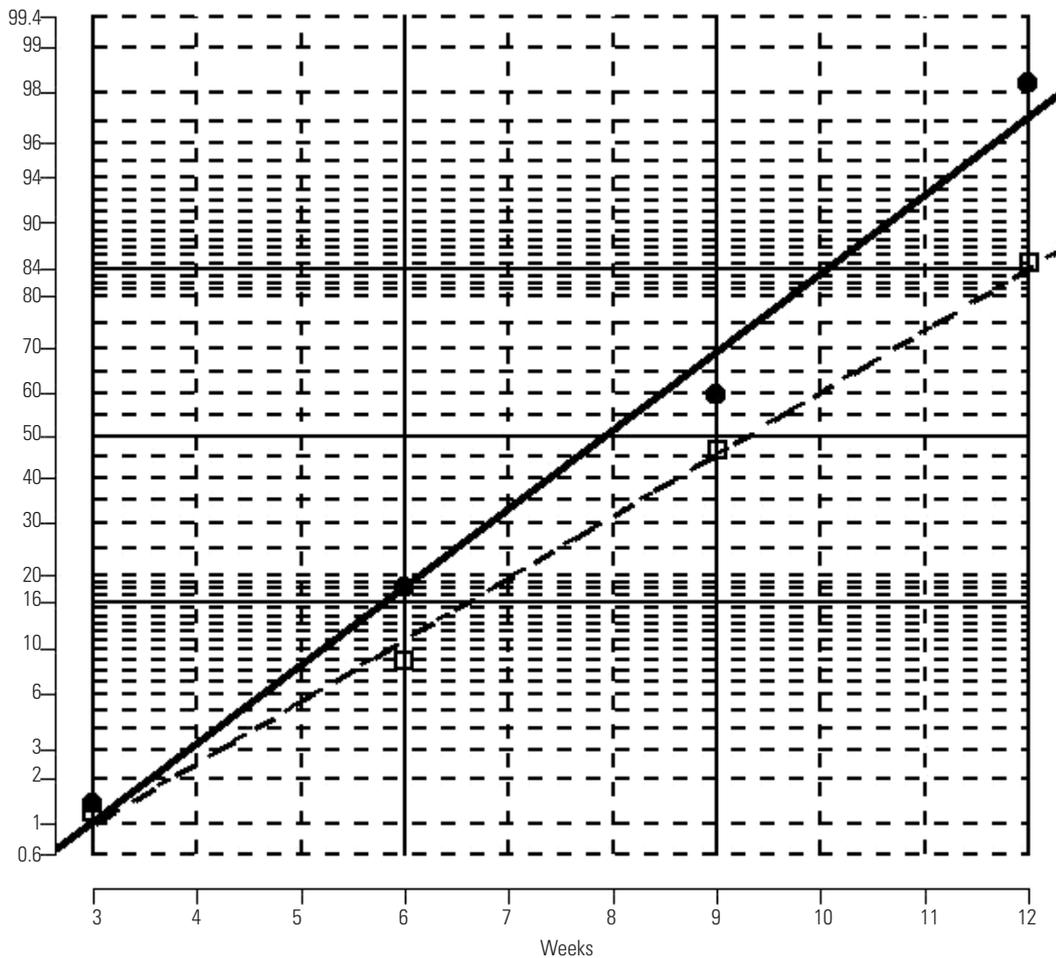
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Fig. 3
Example of regression lines from the percentages of cured accumulated mice obtained with the data shown
in Table I for the reference *(REF)* strain (data represented by circles; regression represented as a plain line)
or problem *(TEST)* strain (data represented by squares; regression represented as a dashed line)

In the example shown in Figure 3, the RT_{50} values are estimated at eight weeks for the *REF* vaccine and 9.2 weeks for the *TEST* vaccine.

The confidence interval for a given RT_{50} value can be calculated graphically by means of the deviation factor f_D of the Litchfield and Wilcoxon nomogram 2 (2). Alternatively, the f_D confidence interval can be calculated mathematically by the formula: $f_D = S^E$, where S is the exponential of the inverse of the regression slope and E is the exponent. For this, S and E values should be determined as follows:

$$S = \frac{\dfrac{RT_{84}}{RT_{50}} + \dfrac{RT_{50}}{RT_{16}}}{2}$$

where RT_{16} and RT_{84} are the estimated values of time for 16% and 84% of cured accumulated mice, respectively, to be

determined graphically over the regression line previously plotted on the modified probit paper, similarly to the RT_{50} value;

$$E = \frac{2.77}{\sqrt{N'}}$$

where 2.77 is a constant and N' the total number of cured mice included in the interval from 16% to 84% of cured accumulated mice (in the Bonet-Maury *et al.* [2] method, the f_D value is determined graphically using nomogram 2 of Litchfield and Wilcoxon, which is no longer required since the corresponding formula is programmable using any scientific calculator).

In the previous example (Table I), the values of the parameters required for these f_D calculations and the f_D values are:

– for the *REF* vaccine: RT_{16} = 5.9 weeks; RT_{50} = 8 weeks; RT_{84} = 10 weeks, leading to S = 1.30; N' = 16 mice, leading to E = 0.69; f_D = 1.19

– for the *TEST* vaccine: $RT_{16} = 6.6$ weeks; $RT_{50} = 9.2$ weeks; $RT_{84} = 12$ weeks, leading to $S = 1.35$; $N' = 16$ mice, leading to $E = 0.69$; $f_D = 1.23$.

In conclusion, in this example, the RT_{50} confidence interval is 8 ± 1.19 weeks (i.e. between 6.81 and 9.19 weeks) for the *REF* vaccine and 9.2 ± 1.23 weeks (i.e. between 7.97 and 10.43 weeks) for the *TEST* vaccine.

Statistical comparison of the RT_{50} obtained for the *TEST* vaccine with that obtained for the *REF* vaccine according to Bonet-Maury *et al.* (2) includes two steps. Firstly, the parallelism of the two regression lines must be tested to validate the parallel line assay. This can be performed either 'visually' or by estimation of slopes b_j and limit values σ_j, using the Litchfield and Fertig approximation method (2), as follows:

$$b_j = \frac{1}{(RT_{84},j - RT_{50},j)}$$

$$\sigma_j = \frac{4}{L \times \dfrac{K}{(K-1)} \times \sqrt{\dfrac{N'_j}{2}}},$$

where L is the range of time tested ($L = 12 - 3 = 9$ weeks), K is the number of points (slaughtering times) for defining the regression line ($K = 4$), and N'_j is the total number of cured mice included in the interval from 16% to 84% of cured accumulated mice for strain j (in the example, $N'_{ref} = N'_{test} = 16$). To validate the parallelism, the two RT_{50} values can be compared only if the slope limit values $b_j \pm \sigma_j$ for both strains are confluent.

The applied example evaluating parallelism by estimation of the slopes and the standard deviations using the Litchfield and Fertig method for the *REF* and the *TEST* vaccines described above is:

– for the *REF* vaccine: $b = 0.5$ and $\sigma_b = 0.12$

– for the *TEST* vaccine: $b = 0.36$ and $\sigma_b = 0.12$.

Consequently, in this example, the limit values of the slopes are (0.38; 0.62) for the *REF* vaccine and (0.24, 0.48) for the *TEST* vaccine. The *REF* and *TEST* vaccines can be compared since the corresponding limit values of their slopes are confluent (2).

Final comparison of the RT_{50} values determined from the parallel regression lines is performed by estimating the following two parameters:

$$r = RT_{50\ ref} - RT_{50\ test}$$

and

$$fr = \exp\left(\sqrt{\ln(f_{D,ref})^2 + \ln(f_{D,test})^2}\right)$$

where $f_{D,ref}$ is the f_D value previously obtained for the *REF* strain and $f_{D,test}$ is the f_D value previously obtained for the *TEST* strain (in the Bonet-Maury *et al.* method (2), the fr value is determined graphically using nomogram 4 of Litchfield and Wilcoxon, which is no longer required since the corresponding formula is programmable using any scientific calculator).

Two RT_{50} values are statistically different when the r value is higher than the fr value ($r > fr$). In the example described above, the r and fr values for the RT_{50} of both *REF* and *TEST* vaccine strains are:

$r = 9.2 - 8 = 1.2$ and $fr = 1.31$.

No significant differences in the RT_{50} of the *REF* and *TEST* strains of the example were observed. Thus, the *TEST* vaccine has an adequate RT_{50} value and successfully passes the quality control test in the mouse model.

An alternative method: Rev 2, a dedicated internet interface

This section presents an alternative statistical method for determining the RT_{50} of anti-*Brucella* vaccines, which is easy to use and currently available free of charge on the internet (www.afssa.fr/interne/rev2.html).

The proposed statistical model uses a general linear model (GLM) for binomial family with a probit link function for the best fit of the experimental data (16). This GLM is based on the same biological assumptions used by Bonet-Maury *et al.* (2) regarding the curing process.

A specific HTML – JAVA SCRIPT programme (named Rev 2) was developed, which submits data to a web-based interface of statistical software which is available free via the internet (15). Those interested in utilising this method only need a connected web browser to use this version. The Rev 2 interface includes precise and easy instructions for use (Fig. 4), making the procedure very simple: the user must enter the name of both the reference and test (problem) vaccines, and the number of mice observed as cured at each slaughter time point. By default, the slaughter time points are three, six, nine and twelve weeks and the number of treated mice at each time point is 8. If any animal dies during the experiment, the corresponding 'treated' cell must be modified. Note that Rev 2 includes an option that provides the details of the statistical procedure in the output window (more details can be found at the website: www.afssa.fr/interne/rev2.html).

The results provided in the output of the Rev 2 programme include the following (Fig. 5):

a) a summary of the entered data (not shown) where the user should verify that the corresponding values were correctly entered

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Fig. 4
Example of the internet interface for determining the recovery time 50 (RT_{50}) of two anti-*Brucella* vaccines, using as input data the rough values indicated in Table I

b) an independent analysis for each vaccine strain

c) a global analysis for both strains; this analysis uses a unique slope β to estimate both RT_{50} values

d) a graphical representation of lines, which enables the fit of the model to be checked graphically (not shown).

The user should verify in the output that all the items 'validity of the model' indicate 'the model could fit' or 'the model could be valid'. Alternatively, the statistical assumptions used are not valid, and the data can definitely not be exploited. Two tests are provided for the comparison of the RT_{50} corresponding to H_0 hypotheses Eq. 2 and Eq. 3. These tests should be concordant. If this is not the case, this means that the degree of signification of the test is very close to the 5% threshold: the equality of both RT_{50} should then be considered with caution. If the model is valid, the best estimate of the RT_{50} values (and their confidence limits) are those proposed in the 'global analysis' section.

Statistical power of the experimental design

This experimental design is adequate if significant differences are detected between two strains that are actually different. The Type I error (often denoted α error) of the procedure, i.e. the probability that the procedure will identify two strains as significantly different when they are not, and the statistical power of the procedure, i.e. the power of the procedure to identify two strains as significantly different when they actually are, will be assessed in this section. Estimation of these parameters cannot be determined using the above-described

❶ **Independant analysis for ref**

Validity of the model: The model could fit ①

Estimate CIinf CIsup ②

RT_{50} 8.1966 6.591028 9.855382

❷ **Independant analysis for test**

Validity of the model: The model could fit ③

Estimate CIinf CIsup ④

RT_{50} 9.52407 7.733454 12.16704

❸ **Global analysis**

Validity of the model: The model could be valid ⑤

RT_{50} for REF

Estimate CIinf CIsup ⑥

RT_{50} 8.224618 6.637563 9.867885

RT_{50} for TEST

Estimate CIinf CIsup ⑦

RT_{50} 9.453317 7.879444 11.20448

Relative potency for Tested ⑧

Estimate Fid. limit inf Fid. limit sup

Relative potency -1.228699 -3.600805 1.022324

COMPARISON OF STRAINS ⑨

Deviance test results: Strains are not different

Relative potency test: Strains are not different

❶ Independent analysis for the reference (*REF*) strain.①: validity of the model (the probit model could be valid for this strain);②: recovery time 50 (RT_{50}) estimate (8.2 weeks) and confidence interval (6.6; 9.9 weeks)
❷ Independent analysis for the *TEST* strain.③: validity of the model (the probit model could be valid for this strain);④: RT_{50} estimate (9.5 weeks) and confidence interval (7.7; 12.1 weeks)
❸ Global analysis (this model uses a unique slope for both strains).⑤: validity of the comparison of strain (the global parallel line assay assumptions could be valid); ⑥ RT_{50} estimate (8.2 weeks) and confidence interval (6.6; 9.8 weeks) for the *REF* strain using a unique slope for both vaccines; ⑦ RT_{50} estimate (9.5 weeks) and confidence interval (7.9; 11.2 weeks) for the *TEST* strain; ⑧Relative potency estimate (–1.2) and fiducial limits (–3.6; 1.02), since the value 0 is in the interval, the RT_{50} of both strains are not statistically different; ⑨: results of two tests for strain comparison (both tests are concordant: the RT_{50} of both strains are not statistically different). The main output to be checked is shown in bold

Fig. 5
Rev 2 output and interpretation

graphical method, while the Rev 2 algorithm allows both to be evaluated.

For this purpose, a RT_{50} value of 7.9 weeks for a 95% confidence interval of 6.3-9.4 weeks (adapted from the European Pharmacopoeia [19]) for the *REF* strain was used as an example. Recovery time 50 values equal to the reference strain (7.9 weeks) or one, two or three weeks lower than that of the reference strain (6.9, 5.9 or 4.9 weeks, respectively) were tested and the results are shown in Table II. The Type I error is about 5% (6% for the deviance test and 5% for the relative

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Table II
Type I error and power according to the differences (in weeks) between the recovery time 50 (RT_{50}) of the reference and test vaccine strains

| Parameter	RT_{50} difference (weeks)	Deviance test	Relative potency test
Type I error	0	0.06	0.05
	1	0.22	0.18
Power	2	0.61	0.56
	3	0.90	0.88

potency test, respectively), meaning that in 5% of the experiments, the protocol leads to identification of significant differences between strains when they are actually identical. This has to be considered adequate since all statistical tests are performed with a theoretical Type I error of 5%. The statistical power of the deviance test is 22%, 61%, and 90% for a tested strain with a $RT_{50\ TEST}$ of one, two or three weeks lower than the $RT_{50\ REF}$ respectively. This means that the method will not detect significant differences in 78%, 39% and 10% of the experiments when the RT_{50} of the problem vaccines are one, two or three weeks lower than the RT_{50} of the reference vaccine, respectively. Despite this, the deviance test is somewhat less powerful than the relative potency test (18%, 56% and 88%, respectively).

Discussion

Vaccination of small ruminants and cattle with classical *B. melitensis* Rev 1 and *B. abortus* S19 live vaccines has been demonstrated as the only practical way to control brucellosis in endemic situations (1, 13). Accordingly, for vaccination campaigns to be successful, good quality commercial batches of both vaccines must be available. Vaccine control based exclusively on examination of classical microbiological markers has been proven to be insufficient to guarantee the biological quality of the vaccines (3, 5, 6, 8, 9, 14). Inadequate manipulation of Rev 1 and S19 strains during manufacture, which is usually based on the seed lot system (18, 19), can result in a loss of biological activity for both vaccines. Thus, control of residual virulence and immunogenicity has to be performed regularly in the mouse model to assess vaccine quality (18, 19). Estimation of the RT_{50} has been shown to be a useful tool for demonstrating that the biological quality of both vaccines is adequate (3, 4, 5, 6, 7, 8, 9, 12, 14, 20, 21, 22). The statistical procedure currently recommended as the reference method by the OIE (18) and the European Pharmacopoeia (19) is a modification of the Bonet-Maury *et al.* method (4). This reference graphical procedure has important practical drawbacks, namely, the complexity of the method, the need for excessive manipulation of data and the subsequent accumulation of potential human errors. This is particularly evident when calculating the accumulated data of Reed-Münch and the parameters of the equations substituting the obsolete nomograms of Litchfield and Wilcoxon. Additional difficulties lie in the need to subjectively draw the regression lines over manually modified probit paper, which is not readily and widely available. These facts have limited the extensive use of the mouse model for controlling the biological quality of both S19 and Rev 1 vaccines and only very few countries regularly apply this control method. In addition to these practical drawbacks, the graphical method also presents some statistical inconsistencies since the Reed and Münch method (23) used is based on an intuitive accumulating theory, which has no adequate scientific basis (10, 11) and leads to a statistical bias (Fig. 6). Moreover, the adjustment of a regression line to observations using the classical least squares method (intuitively used in the graphical fit of the regression line), assumes that the variance at each point is the same while this is not the case in a probit model (16). In the graphical method, the points of probit values placed inside the interval 5%-95% of cured accumulated mice should have a higher weight for a best fit of the hand-plotted regression line. However, an adequate regression fit should employ a weighted least squares method, using weights estimated as the inverse of the variance for each observation. As an example, considering data from the *REF* strain in Table I, the estimated weights of approximately two, six, seven and two for variances of the percentage of cured accumulated mice should be applied for three-, six-, nine- and twelve-week slaughtering time points, respectively. The use of these weights renders impossible the use of a correct manual regression fit, which is essential for the correct performance of the RT_{50} statistical method.

The graphical method currently accepted as the reference method by the OIE and the European Pharmacopoeia is a method suitable for testing the residual virulence of live anti-*Brucella* vaccines. However, given the complexity and practical drawbacks of the method, the mouse model is regularly applied in only very few countries to assess the biological quality of S19 and Rev 1 vaccines. More recent approaches such as the GLM model have been described (16) for more rigorous analysis of these types of experiments. While using the same underlying biological assumptions, GLM presents the advantage of being fully implemented in classical statistical packages. In fact, a computerised version of the reference method using specialised software (24) has been demonstrated to be equivalent to the graphical procedure for RT_{50} calculation (14) (additional information on the computerised version of the reference method is available from the Servicio de Investigación Agroalimentaria in Zaragoza, Spain [mjgrillo@aragob.es]). However, this computerised version also uses the Reed-Münch method with Bartlett's cunning, to comply with OIE and European Pharmacopoeia recommendations (18, 19) and thus also presents some statistical inconsistencies. Moreover, another important disadvantage of this computerised method is that the software used is complex, not easily available and very expensive.

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a)

b)

c)
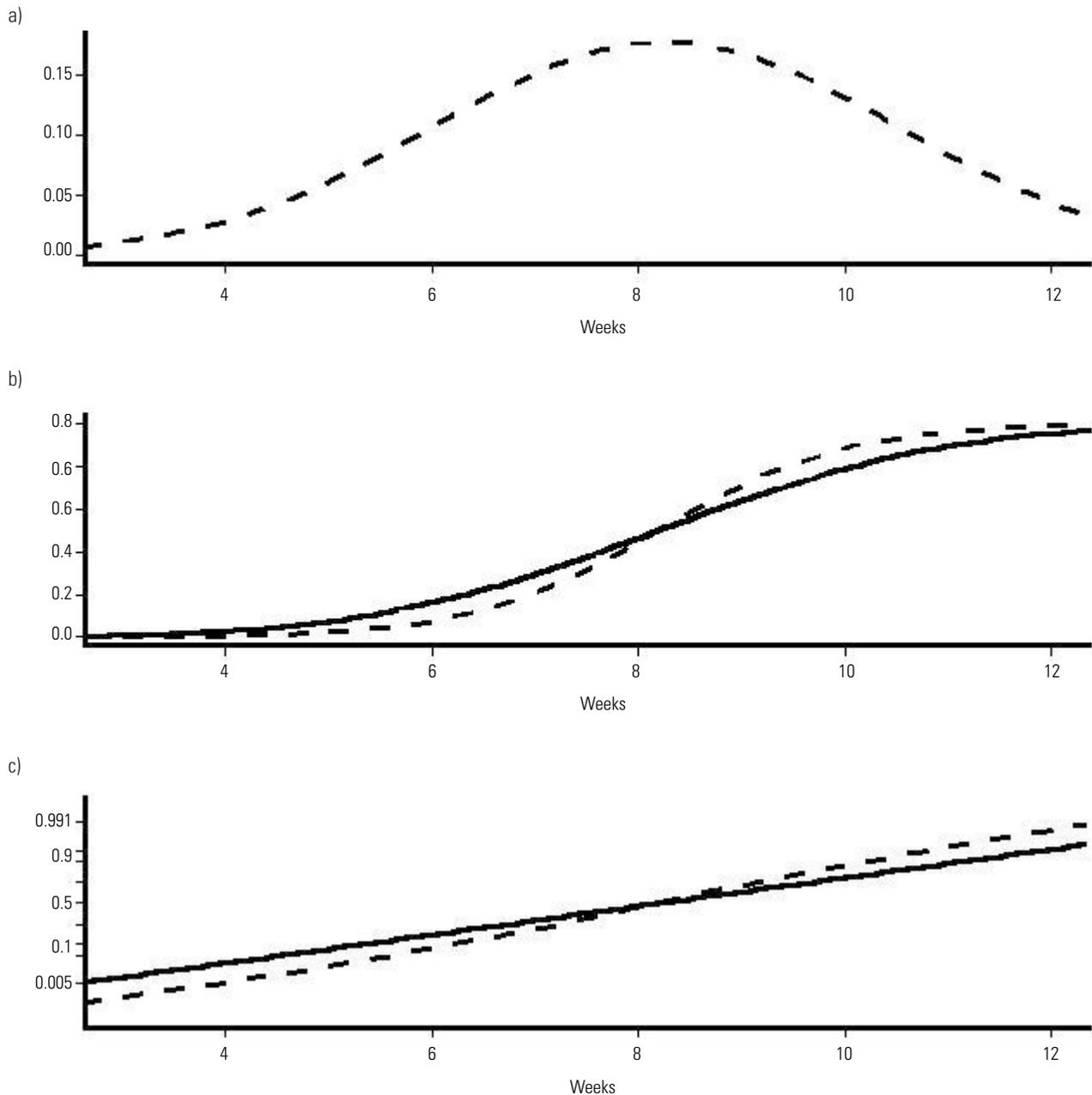
Assuming that the curing time in the mouse population corresponds to the normal distribution provided in figure 6a, the probability for a mouse to be cured at a given time is thus the corresponding cumulative density function represented by the dark line of figure 6b. In a probit scale, this may be represented as the continuous line in figure 6c. For the same distribution of curing time in the mouse population, the Reed and Münch (23) accumulated hypothesis would lead to the cumulative function provided as a dashed line in figure 6b. In a probit scale, this results in the regression line provided in the dashed line of figure 6c. While the RT_{50} is correctly estimated, the confidence interval of the RT_{50} will be sharper artificially, since the slope of this regression line is greater than that of the correct line

Fig. 6
Illustration of the bias from the Reed and Münch method (23) using the tolerance interpretation

In contrast, the internet is now accessible to most biologists world-wide and a statistical interface such as Rev 2, available free of charge on the web, could greatly facilitate RT_{50} calculations for interested biologists. Considering the wide availability of the internet, the Rev 2 interface, being easier to use and statistically more consistent than the method accepted as the reference, may contribute to more widespread use of the mouse model for controlling the quality of Rev 1 and S19 anti-*Brucella* vaccines.

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Procédures statistiques pour le calcul de la virulence résiduelle des vaccins préparés avec la souche 19 (S19) de *Brucella abortus* et la souche Rev. 1 de *Brucella melitensis* chez la souris : fondements théoriques et applications pratiques

R. Pouillot, M.J. Grilló, J.L. Alabart, B. Garin-Bastuji & J.M. Blasco

Résumé
Le contrôle régulier de la qualité biologique des vaccins vivants préparés avec la souche 19 (S19) de *Brucella abortus* et la souche Rev. 1 de *B. melitensis* est indispensable pour garantir une gestion efficace de la brucellose des ruminants dans les pays contaminés. Les procédures de référence préconisées par l'OIE (Organisation mondiale de la santé animale) et la Pharmacopée européenne comprennent la détermination de la virulence résiduelle, exprimée comme le rapport entre le temps moyen de récupération (RT_{50}) du vaccin (problème) testé dans un modèle murin de référence et le RT_{50} des souches de référence correspondantes dans la même épreuve. La procédure statistique sous-jacente se fonde sur un parallélisme des lignes de régression et un modèle probit classique. En réalité, la procédure recommandée pour le calcul du RT_{50} s'inspire d'une méthode graphique qui n'a jamais fait l'objet d'une description exhaustive. Les auteurs expliquent en détail cette méthode graphique en vue de la rendre compréhensible et accessible à tous les biologistes intéressés. Les lourdeurs de la procédure expliquent pourquoi très peu de laboratoires l'utilisent régulièrement pour se conformer aux recommandations de l'OIE et de la Pharmacopée européenne. Étant donné que cette méthode graphique de référence présente par ailleurs des incohérences statistiques, une interface conçue spécialement pour l'Internet a été mise en point pour calculer le RT_{50}. Cette interface est exploitable librement sur la toile (www.afssa.fr/interne/rev2.html).

Mots-clés
Contrôle de la qualité – Méthode de Reed-Münch – Modèle linéaire généralisé – Temps moyen de récupération – Vaccin vivant à Brucella – Virulence résiduelle.

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Fundamentos teóricos y aplicaciones prácticas de los procedimientos estadísticos para calcular la virulencia residual en el ratón de vacunas elaboradas con la cepa 19 de *Brucella abortus* (S19) y de la cepa Rev. 1 de *Brucella melitensis*

R. Pouillot, M.J. Grilló, J.L. Alabart, B. Garin-Bastuji & J.M. Blasco

Resumen
Para hacer frente con éxito a la brucelosis de los rumiantes en los países afectados, es indispensable controlar regularmente la calidad biológica de las vacunas vivas elaboradas con la cepa 19 (S19) de *Brucella abortus* y la cepa Rev. 1 de *B. melitensis*. Los protocolos de referencia recomendados por la OIE (Organización mundial de sanidad animal) y la Farmacopea Europea prescriben

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el cálculo de la virulencia residual, que se expresa como el tiempo de recuperación 50 (RT_{50}) de la vacuna problema en un modelo de referencia en el ratón comparado con el RT_{50} de las correspondientes cepas de referencia en el mismo ensayo. El procedimiento estadístico aplicado se basa en un ensayo de líneas paralelas y un modelo probit clásico. En la práctica, el protocolo que actualmente se recomienda para calcular el RT_{50} deriva de un método gráfico que nunca se ha descrito en detalle. En este artículo los autores ofrecen una descripción completa de ese método gráfico para que resulte comprensible y accesible a todos los biólogos interesados. El procedimiento resulta un tanto enrevesado, y son muy pocos los laboratorios que aplican de modo sistemático las recomendaciones de la OIE y la Farmacopea Europea. Además, teniendo en cuenta que este método gráfico de referencia presenta ciertas anomalías estadísticas, se ha elaborado y colocado en la Web una interfaz Internet dedicada a la cuestión que ofrece la posibilidad de efectuar gratuitamente cálculos del RT_{50} (www.afssa.fr/interne/rev2.html).

Palabras clave
Control de calidad – Método de Reed-Münch – Modelo lineal generalizado – Tiempo de recuperación 50 – Vacuna viva de Brucella – Virulencia residual.

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References

1. Blasco J.M. (1997). – A review of the use of *Brucella melitensis* Rev 1 vaccine in adult sheep and goats. *Prev. vet. Med.*, **31**, 275-283.

2. Bonet-Maury P., Jude A. & Servant P. (1954). – La mesure statistique de la virulence et de l'immunité : application à l'étude de la virulence du vaccin typhique et à la mesure du pouvoir immunisant des vaccins antityphoïdiques. *Rev. Immunol.*, **18** (1), 21-49.

3. Bosseray N. (1985). – Quality control of four Rev 1 anti-*Brucella* vaccines. *In Brucella melitensis* (J.M. Verger & M. Plommet, eds). M. Nijhoff, Lancaster, 229-236.

4. Bosseray N. (1991). – *Brucella melitensis* Rev 1 living attenuated vaccine: stability of markers, residual virulence and immunogenicity in mice. *Biologicals*, **19**, 355-363.

5. Bosseray N. (1992). – Le vaccin Rev 1 : dérive des caractères d'immunogénicité et de virulence indépendante des marqueurs classiques. *In* Prevention of brucellosis in Mediterranean countries (M. Plommet, ed.). Pudoc Scientific Publishers, Wageningen, 182-186.

6. Bosseray N. (1992). – Control methods and thresholds of acceptability for anti-*Brucella* vaccines. *Dev. biol. Standard.*, **79**, 121-128.

7. Bosseray N. & Plommet M. (1976). – Transformation normalisant la distribution du nombre de *Brucella* dans la rate de souris inoculées par voie intrapéritonéale. *J. biol. Standard.*, **4**, 341-351.

8. Bosseray N., Plommet M. & Plommet A.M. (1984). – Theoretical, practical, and statistical basis for general control method of activity for anti-*Brucella* vaccines. *Dev. biol. Standard.*, **56**, 257-270.

9. Bosseray N. & Plommet M. (1990). – *Brucella suis* S2, *Brucella melitensis* Rev 1 and *Brucella abortus* S19 living vaccines: residual virulence and immunity induced against three *Brucella* species challenge strains in mice. *Vaccine*, **8**, 462-468.

10. Finney D.J. (1964). – Statistical method in biological assay, 2nd Ed. Charles Griffin and Company Limited, London and High Wycombe, 668 pp.

11. Finney D.J. (1978). – Statistical method in biological assay, 3rd Ed. Charles Griffin and Company Limited, London and High Wycombe, 508 pp.

12. Garín-Bastuji B. (1992). – Contrôle officiel du vaccin Rev 1 en France. Modalités, critères, sanction. *In* Prevention of brucellosis in Mediterranean countries (M. Plommet, ed.). Pudoc Scientific Publishers, Wageningen, 176-181.

13. Garín-Bastuji B., Blasco J.M., Grayon M. & Verger M. (1998). – *Brucella melitensis* infection in sheep – present and future. *Vet. Res.* **29**, 255-274.

14. Grilló M.J., Bosseray N. & Blasco J.M. (2000). – *In vitro* markers and biological activity in mice of seed lot strains and commercial *Brucella melitensis* Rev 1 and *Brucella abortus* B19 vaccines. *Biologicals*, **28** (2), 119-127.

15. Ihaka R. & Gentleman R. (1996). – R: a language for data analysis and graphics. *J. Comput. Graph. Stat.*, **5**, 299-314.

16. McCullagh P. & Nelder J.A. (1989). – Generalized linear models, 2nd Ed. Chapman and Hall, London, 511 pp.

17. Morgan B.T.J. (1998). – Quantal response models. *In* Encyclopedia of biostatistics (P. Armitage & T. Colton, eds). Wiley and sons, Chichester, 3618-3625.

18. OIE (World organisation for animal health) (2000). – Manual of standards for diagnostic tests and vaccines, 4th Ed. OIE, Paris, 957 pp.

19. Pharmacopée Européenne (2001). – Vaccin vivant cryodesséché de la brucellose (*Brucella melitensis* Souche Rev 1) pour usage vétérinaire. *In* Pharmacopée Européenne, 4th Ed. Conseil de l'Europe, Strasbourg, 2459.

20. Plommet M. (1995). – Living vaccines: virulence, immunogenicity/protection, and safety: historical, theoretical, and practical considerations applied to the *Brucella melitensis* Rev 1 vaccine. *In* Round table on the use of Rev 1 vaccine in small ruminants and cattle (B. Garín-Bastuji & A. Benkirane, eds), 21-22 September 1995, Maisons-Alfort. Centre National d'Etudes Vétérinaires et Alimentaires, Maisons-Alfort, 7-18.

21. Plommet M. & Bosseray N. (1977). – Le contrôle des vaccins antibrucelliques par dénombrement des *Brucella* dans la rate de souris, vaccinées ou non, inoculeés par voie intrapéritonéale. *J. biol. Standard.*, **5**, 261-274.

22. Plommet M. & Bosseray N. (1984). – Propositions pour une méthode générale de contrôle d'activité des vaccins antibrucelliques. *Dev. biol. Standard.*, **56**, 247-255.

23. Reed L.J. & Münch H. (1938). – A simple method of estimating 50 percent end-points. *Am. J. Hyg.*, **27**, 493.

24. SAS Institute Inc. (1989). – SAS/STAT® user's guide, version 6, 4th Ed., Vol. 2. NC: SAS Institute Inc., Carry, 943 pp.