**COMPARISON OF SEROLOGICAL AND MILK TESTS FOR BOVINE BRUCELLOSIS USING A MONTE CARLO SIMULATION MODEL**

A. Giovannini, A. Conte, A. Petrini, L. La Porta, D. Nannini & V. Caporale

1 Istituto Zooprofilattico Sperimentale dell’Abruzzo e del Molise «G. Caporale», Teramo - Italia
2 Azienda Sanitaria Locale 3 Centro Molise, Campobasso - Italia

**Introduction**

Article 4 of the Sanitary and Phytosanitary Agreement (SPS) of the World Trade Organization (WTO) states that:

«Members shall accept the sanitary or phytosanitary measures of other Members as equivalent, even if these measures differ from their own or from those used by other Members trading in the same product, if the exporting Member objectively demonstrates to the importing Member that its measures achieve the importing Member’s appropriate level of sanitary or phytosanitary protection. For this purpose, reasonable access shall be given, upon request, to the importing Member for inspection, testing and other relevant procedures».

European Union (EU) Directive 97/12/EC allows the trade of cattle within the EU of animals originating from an ‘officially brucellosis-free herd’. To qualify for this status, a number of different programmes must be implemented. Each EU Member Country is free to decide which procedure to use to qualify herds. The authors conducted a study to compare the merits and costs of testing programmes given in the Directive and of some alternative testing strategies. The effectiveness of testing programmes was evaluated by a Monte Carlo simulation model. Programmes listed in the Directive do not appear to have identical sensitivity and specificity. Simulations of the programmes showed that milk testing may be more effective and efficient than blood testing to identify infected herds. Results indicated that it could be advisable that legislation, rather than defining very detailed procedures both for laboratory tests and testing programmes, should establish minimal requirements in terms of efficacy of testing procedures (i.e. the probability of detecting an infected herd).

**Materials and Methods**

In accordance with Council Directive 97/12/EC, a cattle herd can be classified as an «officially brucellosis free herd» when the herd has given negative results to the

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**SUMMARY**

European Union (EU) Directive 97/12/EC allows the trade of cattle within the EU of animals originating from an ‘officially brucellosis-free herd’. To qualify for this status, a number of different programmes must be implemented. Each EU Member Country is free to decide which procedure to use to qualify herds. The authors conducted a study to compare the merits and costs of testing programmes given in the Directive and of some alternative testing strategies. The effectiveness of testing programmes was evaluated by a Monte Carlo simulation model. Programmes listed in the Directive do not appear to have identical sensitivity and specificity. Simulations of the programmes showed that milk testing may be more effective and efficient than blood testing to identify infected herds. Results indicated that it could be advisable that legislation, rather than defining very detailed procedures both for laboratory tests and testing programmes, should establish minimal requirements in terms of efficacy of testing procedures (i.e. the probability of detecting an infected herd).

**KEYWORDS**

Brucellosis, Cattle, Complement fixation test, Enzyme-linked immunosorbent assay, Equivalence, Milk, Milk ring test, Rose Bengal test.

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**COMPARAZIONE DI PROVE SIEROLOGICHE E SUL LATTE MEDIANTE UN MODELLO DI SIMULAZIONE MONTE CARLO**

Riassunto

La Direttiva 97/12/EC permette il commercio di bovini all'interno dell'Unione Europea (UE) purché provengano da "allevamenti ufficialmente indenni" (AUI) da brucellosi. La qualifica di AUI si basa su una serie di programmi di controllo differenti. Ciascun Paese membro dell'UE ha deciso la sua procedura di accreditamento degli allevamenti. Il presente studio è stato svolto per valutare l'equivalenza e i costi dei diversi programmi di controllo degli allevamenti previsti dalla direttiva e di alcuni programmi di controllo alternativi non previsti dalla direttiva stessa. L'efficacia dei programmi di controllo è stata valutata tramite un modello di simulazione Monte Carlo. I programmi previsti nella direttiva non sembrano avere sensibilità e specificità equivalenti. Infatti, le simulazioni effettuate mostrano che le prove sul...
following:

a) two serological tests, based on the serum agglutination test (SAT), Rose Bengal test (RBT), complement fixation test (CFT), enzyme-linked immunosorbent assay (ELISA), the buffered Brucella antigen test, plasma ring test or plasma agglutination test, of the entire herd, at an interval of more than three months and less than twelve months (Programme A); or

b) three milk ring tests (MRT) at quarterly intervals, followed by a serological test at least six weeks later (Programme B).

A cattle herd will maintain its «officially brucellosis-free herd» status if negative results are obtained from either:

a) two serological tests as listed above, conducted at an interval of at least three months and not more than six months (Programme A); or

b) three milk ring tests (Programme C); or

c) three milk-ELISAs (Programme D) performed at intervals of at least three months.

If positive results are obtained from Programmes C and D, individual serological testing is required to detect the infected animals.

The only procedure applied in Italy is Programme A, based on RBT as the screening test and CFT as the confirmatory test. Due to the simplicity and low cost of the MRT, a comparison was made of this test with serological tests, including milk testing. The same type of evaluation could be performed for purposes of international trade, for the evaluation of equivalent measures applied in different countries.

Five additional procedures, not considered by Council Directive 97/12/EC were also simulated, as follows:

1) three milk-ELISAs followed by a serological test (Programme E);

2) four MRTs (Programme F);

3) five MRTs (Programme G);

4) four milk-ELISAs (Programme H);

5) five milk-ELISAs (Programme I).

The quality of antigens used and herd factors, such as the percentage of milking cows in the herd, affect milk test performance. In the same way, the quality of antigens influences the performance of serological tests. Therefore, the evaluation was performed using real data describing local conditions.

The testing programmes were compared in three different scenarios, as follows:

a) infected herds and optimal fertility (i.e. lactation: 305 days; delivery-conception: 90 days); the aim of this scenario was to evaluate herd sensitivity to milk testing in optimal conditions;

b) infected herds and the fertility level observed in a province of southern Italy (i.e. lactation: 294 days; delivery-conception: 157 days); the aim of this scenario was to evaluate herd sensitivity to milk testing in sub-optimal herd fertility conditions

c) non-infected herds and the fertility level observed in a province of southern Italy; the aim of this scenario was to evaluate herd sensitivity to milk testing in sub-optimal herd fertility conditions.

The quality of antigens used and herd factors, such as the percentage of milking cows in the herd, affect milk test performance. In the same way, the quality of antigens influences the performance of serological tests. Therefore, the evaluation was performed using real data describing local conditions.

The variables used in the model running 10,000 iterations. The effectiveness of testing programmes was evaluated by a Monte Carlo simulation model, using a detailed Monte Carlo simulation model, running 10,000 iterations.
were as follows:

a) sensitivity and specificity of tests used, both in individual animals and in bulk milk, are derived from previous investigations (1, 2);
b) number of herds and herd size are those resulting from the cattle identification and registration system in a region in southern Italy;
c) prevalence of infection by herd size is derived from routine serological testing of cattle herds in the same region;
d) fertility data and duration of milking were obtained from the data of an association of breeders in a province of southern Italy.

For each variable, the most appropriate statistical distribution was used for the implementation of the model (5). In particular, the model was divided into five modules as follows:

1) herd status;
2) milk ring tests;
3) milk-ELISAs;
4) serological tests;
5) summary of outputs.

The description of the model is based on the second scenario, namely: infected herds and the fertility level observed in southern Italy (i.e. lactation: 294 days; delivery-conception: 157 days).

Herd status

The objective of the module was to simulate results of herd testing using the milk-ELISA (i.e. to determine whether infection in the herd is detected by the milk-ELISA test).

The input data were as follows:

• data on milk-ELISA sensiti-vity and specificity based on individual animal data (1)
• experimental data on the highest dilution of milk that gives a positive reaction to the milk-ELISA (1). The cumulative distribution from experimental data was compared to the ratio of simulated values of milking cows/infected cows so as to simulate the ability of the milk-ELISA to detect infection in the herd.

The input data and structure of the module on the MRT are summarised in Table II.

Milk-enzyme-linked immunosorbent assay

The objective of the module was to simulate results of herd testing using the MRT (i.e. to determine whether the herd infection is detected or not by this test).

The input data were as follows:

• MRT sensitivity and specificity on individual animals (1);
• experimental data on the highest dilution of milk that gives a positive reaction to the MRT (1). The cumulative distribution from experimental data was compared to the ratio of simulated values of milking cows/infected cows so as to simulate the ability of the MRT to detect infection in the herd.

The input data and structure of the module on the MRT are summarised in Table II.

Serological testing

The objective of the module was to simulate results of herd serology testing (i.e. to determine whether infection in the herd is detected by serology).

The input data was as follows:

• RBT and CFT sensitivity and specificity (2).

In Italy, RBT and CFT are the most widely used serological tests.
recognised by EU legislation.

The input data and structure of the module on serological tests are summarised in Table IV.

**Simulation of test program**

The previous modules were repeated and different combinations were obtained to reproduce the following programmes:
- two serological tests;
- three MRTs;
- four MRTs;
- five MRTs
- three MRTs and one serological test;
- three milk-ELISAs;
- four milk-ELISAs;
- five milk-ELISAs;
- three milk-ELISAs and one serological test.

**Summary of outputs**

The final module provides the summary of outputs, the objective of which was to combine the results of the previous modules. The structure of the module is summarised in Table V.

**Evaluation of the specificity of testing program**

The specificity of testing programme modules is similar to those developed for sensitivity. Differences are shown in Table VI.

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**Results**

All results are summarised in Table VII and in Figures 1, 2 and 3. Figures 1, 2 and 3 provide the results of Scenario 1 (optimal fertility and infected herds), Scenario 2 (fertility observed in a region of southern Italy and infected herds) and herd specificity in non-infected herds, respectively.

The costs of the various options are shown in Figure 4. The following costs (current prices in Italy) were considered:
- cost of a herd visit to collect either blood or milk samples: € 4.04;
- cost of collecting each blood sample: € 0.57;
- cost of blood sample for RBT testing (including economy of scale applicable to batch testing the entire herd): € 0.07;
- cost of the confirmatory CFT testing (including economy of scale applicable to batch testing): € 0.14;
- cost of milk-ELISA: € 0.19;
- cost of MRT: € 3.33.

In the economic evaluation, the cost of confirmatory serological testing for herds that gave positive results to milk tests is included.

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**Figure 1: Simulation: optimal fertility in infected herds.**

*Figura 1: Simulazione: mandrie infette (fertilità ottimale).*

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l’equivalenza delle misure applicate in Paesi differenti.

Nelle simulazioni sono state incluse anche cinque ulteriori procedure, non considerate dalla direttiva 97/12/EC:
- tre prove ELISA-latte seguite da una prova sierologica (programma E);
- quattro prove dell’anello (Ring Test) (programma F);
- cinque prove dell’anello (Ring Test) (programma G);
- quattro prove ELISA-latte (programma H);
- cinque prove ELISA-latte (programma I).

La qualità degli antigeni usati e fattori inerenti la mandria, come la percentuale di vacche in lattazione, possono influenzare le prestazioni delle prove sul latte. Allo stesso modo, la qualità degli antigeni usati influenza le prestazioni delle prove sierologiche. Pertanto, la valutazione dell’equivalenza è stata effettuata utilizzando dati reali, tratti dalle condizioni locali in cui le prove dovevano essere utilizzate.

I programmi di controllo sono stati confrontati nei seguenti tre scenari:
- mandrie infette e valori ottimali dei parametri di fertilità (cioè, durata della lattazione: 305 giorni; intervallo parto-concepimento: 90 giorni); scopo di questo scenario era quello di valutare le prestazioni delle prove sul latte in mandrie in condizioni ottimali.
- mandrie infette e valori dei parametri di fertilità osservati in una provincia dell’Italia meridionale (cioè, durata della lattazione: 294 giorni; intervallo parto-concepimento: 157 giorni); scopo di questo scenario era quello di valutare la specificità delle prove sul latte in condizioni di fertilità sub-ottimali.
- mandrie non infette e valori dei parametri di fertilità osservati in una provincia dell’Italia meridionale (cioè, durata della lattazione: 294 giorni; intervallo parto-concepimento: 157 giorni); scopo di questo scenario era quello di valutare la specificità delle prove sul latte in condizioni di fertilità sub-ottimali.

Questo scenario non ha importanza dal punto di vista del commercio internazionale, ma permette una migliore valutazione dei programmi di controllo e permette di stimare la parte dei costi delle strategie di controllo.
Programmes B, F and G are more expensive than serological testing, while programmes C, D, H and I are more economical (Fig. 4).

Discussion

Programmes listed in Council Directive 97/12/EC do not appear to offer equivalent sensitivity and specificity values, at least in situations similar to those encountered in Italy as far as fertility is concerned (Table VII). In particular, the sensitivity of herd testing varies from a minimum of 63.2% in Programme C (three MRTs) in cattle populations with a consequent alla carenza di specificità. La sensibilità e la specificità della sierologia, sia a livello individuale che di mandria, sono state considerate indipendenti dalla durata della lattazione e dalla lunghezza del periodo parto-concepimento.

L'efficacia dei programmi di controllo è stata valutata mediante un modello di simulazione Monte Carlo, del quale sono state effettuate 10,000 iterate.

Le variabili usate nel modello e le fonti dei dati sono le seguenti:

a) sensibilità e specificità dei test utilizzati, sia su basi individuali che sul latte di massa, ottenute da precedenti indagini (1, 2)

b) numero di allevamenti e grandezze degli allevamenti, ottenute dal sistema di identificazione e registrazione del bestiame bovino di una provincia dell'Italia meridionale

c) prevalenza di infezione, suddivisa per classi di grandezza dell'allevamento, ricavate dai risultati delle attività correnti di esame sierologico per brucellosi svolte nella stessa provincia

d) dati sulla fertilità e durata del periodo di lattazione, forniti dall'associazione degli allevatori della stessa provincia.

Per ciascuna delle variabili considerate, nel modello è stata utilizzata la distribuzione di probabilità più appropriata (5). In particolare, il modello è stato suddiviso nei cinque moduli seguenti:

1) stato sanitario
2) prove dell'anello (Ring Test)
3) prove ELISA latte
4) prove sierologiche
5) sommario degli output

Per la descrizione del modello si farà riferimento al secondo scenario, cioè mandrie infette e valori dei parametri di fertilità osservati in una provincia dell'Italia meridionale (durata della lattazione: 294 giorni; intervallo parto-concepimento: 157 giorni).

**Stato sanitario**

L’obiettivo del modulo è simulare le distribuzioni di frequenza della prevalenza di infezione negli allevamenti e nel sotto-insieme delle vacche in lattazione di ciascun allevamento.

I dati di input sono:

- distribuzione della grandezza
sub-optimal fertility, to a maximum of 99.9%, in Programme I (five milk-ELISAs) in cattle populations with an optimal level of fertility.

Differences observed in the results obtained from the different strategies in the two scenarios concerning sensitivity seem more marked when comparing tests as opposed to fertility levels. Fertility may affect testing efficacy when small numbers of milk tests are conducted (Programme D in Table VII).

Simulations tend to indicate that the programme based on three milk-ELISAs is in fact the most sensitive testing strategy among those provided by the Directive and is definitely more sensitive than that based on MRT, although the milk-ELISA-based programme can be used for the maintenance of qualification, but not for initial herd qualification, according to EU legislation.

The simulations performed show that milk testing may be more effective and efficient than blood testing to identify infected herds. A programme based on four or more tests of the herd using the milk-ELISA appears to offer much greater sensitivity than classical serological testing and, at the same time, may also prove more economical than serology, despite the cost of confirmatory tests required to cater for the lower specificity of milk testing.

According to EU Council Directive 92/46/EEC, raw milk, heat-treated milk and milk-based products (3) must be sampled at least twice a month for plate counts. Therefore, the cost of field activities for the collection of samples (the bulk of brucellosis testing costs) can be reduced further by using milk samples collected in accordance with the provisions of Directive 92/46/EEC.

In conclusion, results showed that it could be advisable that legislation, rather than defining very detailed procedures both for laboratory tests and testing programmes, should establish minimal requirements for the efficacy of testing procedures (i.e. the probability of detecting an infected herd). Legislation, such as that of EU, which allows for each national competent authority to select a programme from various alternatives seems to be inappropriate if the objective is to have equivalent test results in different Countries or Regions. This is particularly true when conditions in each country or region vary.

A field trial is in progress in a province of southern Italy to verify the forecasts of the model used.

References/Bibliografia

mandria viene rilevata o meno dalla sierologia)

I moduli sopra descritti sono stati ripetuti nelle varie combinazioni necessarie per riprodurre i seguenti programmi di controllo:

- due prove sierologiche;
- tre prove dell’anello;
- quattro prove dell’anello;
- cinque prove dell’anello;
- tre prove dell’anello + una prova sierologica;
- tre prove ELISA-latte;
- quattro prove ELISA-latte;
- cinque prove ELISA-latte;
- tre prove ELISA-latte + una prova sierologica.

**Sommaario degli output**

L’ultimo modulo fornisce il sommario degli output, il cui obiettivo è quello di combinare insieme i risultati dei precedenti moduli.

La struttura del modulo è riassunta nella Tabella V.

**Valutazione della specificità dei programmi di controllo**

I moduli sulla specificità dei programmi di controllo sono simili a quelli sviluppati per la sensibilità. Le differenze sono riportate in Tabella VI.

**Risultati**

Tutti i risultati sono riassunti in Tabella VII e nelle Figure 1, 2 e 3. Le Figure 1, 2 e 3 mostrano rispettivamente i risultati dello Scenario 1 (mandrie infette e valori ottimali dei parametri di fertilità), Scenario 2 (mandrie infette e valori dei parametri di fertilità osservati in una provincia dell’Italia meridionale) e specificità a livello di mandria in allevamenti non infetti (Scenario 3).

I costi delle diverse opzioni di controllo sono rappresentati nella Figura 4. Per il calcolo dei costi, sono stati considerati i seguenti valori (prezzi correnti in Italia):

- costo di un ingresso in allevamento per raccogliere campioni di sangue o di latte: € 4,04;
- costo per la raccolta di ciascun singolo campione di sangue: € 0,57;
- costo dell’esame di un campione di sangue mediante SAR (incluso le economie di scala conseguenti all’esame in blocco di diversi campioni positivi): € 0,14;
- costo di una prova ELISA-latte: € 0,19;
- costo di una prova dell’anello: € 3,33.

Nelle valutazioni economiche è incluso anche il costo degli esami sierologici di conferma per gli allevamenti che hanno dato risultati positivi alle prove sul latte.

I Programmi di controllo B, F e G sono più costosi dell’esame sierologico, mentre i programmi C, D, H ed I sono più economici (Figura 4).

**Discussione**

I programmi previsti dalla Direttiva del Consiglio 97/12/EC, sulla base dei risultati ottenuti, non sembrano avere valori di sensibilità e di specificità equivalenti, almeno in allevamenti con valori dei parametri di fertilità simili a quelli prevalenti in Italia (Tabella VII). In particolare, la sensibilità nell’esame delle mandrie varia da un minimo del 63,2% per il Programma C (tre prove dell’anello) in popolazioni bovine con valori di fertilità sub-ottimali, ad un massimo del 99,6% per il programma I (cinque prove ELISA-latte) in popolazioni bovine con valori di fertilità ottimali.

Le differenze osservate nei risultati delle diverse strategie di controllo, nei due scenari relativi alla sensibilità, sembrano più evidenti tra test differenti piuttosto che tra livelli differenti di fertilità. La fertilità può influenzare l’efficacia del controllo quando il programma prevede l’esecuzione di un piccolo numero di prove sul latte (Programma D in Tabella VII).

Le simulazioni tendono ad indicare che il programma basato su tre prove ELISA-latte è, in realtà, la più sensibile tra le strategie di controllo previste dalla Direttiva ed è decisamente più sensibile di quello basato sulla prova dell’anello. Ciò, a dispetto del fatto che il programma basato sull’ELISA-latte sia previsto dalla legislazione europea solo per la riconferma della qualifica, non per il conferimento iniziale della qualifica all’allevamento.

Le simulazioni dimostrano che l’esame del latte può essere più efficace e più efficiente degli esami sierologici.
Table 1: Structure of the herd status module.

Table 1: Struttura del modulo per lo stato della mandria.

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
</tr>
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<tr>
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<td>Herd size: dairy cows</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>2</td>
<td>Class no.: lower limit</td>
<td>upper limit</td>
<td>frequency</td>
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<td>300</td>
<td>32</td>
<td>2347</td>
<td>718</td>
<td>5</td>
</tr>
</tbody>
</table>

A       B                   C
10 Milk cows
11 Delivery-new pregnancy | 157  |
12 Pregnancy               | 270  |
13 Lactation               | 294  |
14 Cows                    | 5998 |
15 Probability of milking  | =B13/(B11+B12) |
16 Standard error          | =B15*(1-B15)^0.5/B14^0.5 |

A       B       C
21 No. dairy cows | Class no. | No. milking cows |
22 =Round(Risk cumul (B3,C9,C4,C9,H4,H9),0) | Lookup (A22,C3,C9,A3,A9)+1 | Risk binomial (A22,RiskNormal (B15,B16,0,1)) |

D       E       F
21 Tested | Positive | No. infected cows |
22 Lookup | Lookup | Risk binomial (A22,riskBeta (E22+1,D22-E22+1)) |

G       H
21 No. infected milking cows | Control variable |
22 =IF (AND (F22>0,C22>0),RiskHypergeo (C22,F22,A22),0) | =IF (F22>0,1,0) |

The meaning of the variables and the choice of distributions were based on the following rationale:

A22: No. of dairy cows: The number of dairy cows in the herd was extracted from the empirical distribution observed in the study area from which data were collected. This variable is the pivot on which the entire model (i.e. the five modules) is based. Since data were collected on the basis of size classes of pre-defined width, the empirical cumulative distribution was used. Should the data be available in the form of single values (i.e. the entire set of sizes of each herd) an empirical discrete distribution would be more appropriate. The minimal value for the cumulative distribution was taken as equal to zero in order to have a probability greater than zero of extracting herds with one milking cow.

B22: Class No.: This is a dummy variable needed because the Excel lookup function is unable to seek values within a range, but requires defined values.

C22: No. of milking cows: The variable follows a binomial distribution where the number of milking cows is the number of successes and the sample size is the total number of cows in the herd. The probability of being a milking cow, to be used in the binomial distribution, can be given by a truncated normal distribution in order to take into account the variability of the phenomenon. The source of data for the probability of milking derives from what has been observed in a region of southern Italy (cells A11:B16).

F22: No. of infected cows: This variable also follows a binomial distribution where the number of infected cows is the number of successes out of the total number of cows in the herd. The probability of being infected can be represented by a beta distribution with parameters given by input data; in this way, the uncertainty of the estimation is taken into account. Data derive from serological activity performed in the study area: the number of tested animals is the number of animals that were tested in positive herds, subdivided by the herd size class, the number of positives is the number of animals that gave a positive result.

G22: No. of infected milking cows: This variable follows a hypergeometric distribution in which the number of successes in the sample is the number of infected milking cows in the herd. The probability of extracting samples with zero successes, the aim of this variable is to exclude those iterations in which the extracted herd is not infected.
Table II: Structure of the milk ring test module.

<table>
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<th>C</th>
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|    |     |     | Determination of bacterial load. However, the cost of the activities of the field relative to the preselection of the cows (quota of eradication cost of the MRT) should be reduced using the dilution of the milk samples at the detection of the E. coli in the bulk milk of the simulated herd. In conclusion, the results obtained have shown that the legislation should set the minimum requirements in terms of efficacy of the procedure of control (in order to detect the infection of the herd), instead of defining in detail the procedures of control and those for the execution of the laboratory tests. A legislation, as that of the Community, that allows each competent authority of the Member States to choose between various alternatives seems particularly inadequate to obtain an equivalent of the results of the control. This is particularly true when the conditions vary in each country or region.

Currently, a field test is in progress in a province of southern Italy to verify the predictions of the model.

The meaning of the variables and the choice of distributions were based on the following rationale:

A.36: This variable represents the number of infected milking cows (G22) that reacted positively to the MRT when milk was examined undiluted, consequently a binomial distribution with a p equal to the sensitivity of the MRT is the most appropriate. The sensitivity of the MRT can be estimated by a beta distribution taking into account the uncertainty of input data.

B.36: This variable expresses the maximum dilution at which MRT is able to provide a positive result. The source of data is an experiment performed through progressive dilutions of MRT-positive field milk samples in negative milk.

C.36: This variable is the comparison between the maximum dilution given by variable B.36 and the dilution of milk from infected milking cows reacting positive to the MRT in the bulk milk of the simulated herd.
### Table III: Structure of the milk-ELISA test module.

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity (individual basis)</td>
<td></td>
</tr>
<tr>
<td>38</td>
<td>Infected</td>
</tr>
<tr>
<td>39</td>
<td>44</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>Dilution</td>
<td>Frequency</td>
</tr>
<tr>
<td>41</td>
<td>200</td>
<td>1</td>
</tr>
<tr>
<td>42</td>
<td>400</td>
<td>4</td>
</tr>
<tr>
<td>43</td>
<td>800</td>
<td>2</td>
</tr>
<tr>
<td>44</td>
<td>1600</td>
<td>1</td>
</tr>
</tbody>
</table>

### Table IV: Structure of the serological tests module.

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Individual</td>
<td>Dilution</td>
<td>Bulk milk result - TP: is herd infected according to ELISA?</td>
</tr>
<tr>
<td>45</td>
<td>=RiskBinomial (G22, RiskBeta (B39+1,A39-B39+1))</td>
<td>=IF (AND (A46&gt;0,G22&gt;0), (B39+1, A39-B39+1)) (1,A44,A41:A44, C41:C44)</td>
</tr>
<tr>
<td>46</td>
<td>=RiskCumul</td>
<td>IF (B46&gt;A46/G22,1,0),0</td>
</tr>
</tbody>
</table>

The meaning of the variables and the choice of distributions were based on the same rationale as that for the MRT.

### The meaning of the variables and the choice of distributions:

- **A59**: RBT true positive results - This variable represents the number of successes out of n trials (where n is the number of infected cows in the herd), therefore the binomial distribution with a p equal to the sensitivity of the RBT has been used. The sensitivity of the RBT can be estimated by a beta distribution, taking into account the uncertainty of input data.

- **C59**: CFT-positive - This variable follows a binomial distribution in which the number of trials is represented by the infected cows detected by RBT and p is equal to the sensitivity of the CFT. The sensitivity of the CFT can be estimated by a beta distribution, taking into account the uncertainty of input data.

- **D59**: Is the herd infected according to serology? - This variable is the interpretation of a series of results according to the European law.
Table VI: Structure of the summary of outputs module.

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>65</td>
<td>Summary</td>
<td>1st test</td>
<td>2nd test</td>
</tr>
<tr>
<td>66</td>
<td>Is herd+&amp;MRT+?</td>
<td>=IF (AND (H22&gt;0, D36),1,0)</td>
<td>=IF (AND (H22&gt;0, result 2ndMRT),1,0) [1]</td>
</tr>
<tr>
<td>67</td>
<td>Is herd+&amp;m-ELISA+?</td>
<td>=IF (AND (H22&gt;0, D46),1,0)</td>
<td>=IF (AND (H22&gt;0, result 2nd ELISA),1,0) [1]</td>
</tr>
<tr>
<td>68</td>
<td>Is herd+&amp;serology+?</td>
<td>=IF (AND (H22&gt;0, D59),1,0)</td>
<td>=IF (AND (H22&gt;0, result 2nd serology),1,0) [1]</td>
</tr>
<tr>
<td>69</td>
<td>Infected?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>70</td>
<td>=IF (H22=1,1,“”)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>71</td>
<td>Detection of infection</td>
<td>3 tests</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>Serology</td>
<td>=IF (A70=1, IF((B68+C68)&gt;0,0,1),0)</td>
<td></td>
</tr>
<tr>
<td>73</td>
<td>MRT</td>
<td>=IF (A70=1, IF((B66+C66)&gt;0,0,1),0)</td>
<td></td>
</tr>
<tr>
<td>74</td>
<td>ELISA</td>
<td>=IF (A70=1, IF((B67+C67)&gt;0,0,1),0)</td>
<td></td>
</tr>
<tr>
<td>75</td>
<td>Serology + MRT</td>
<td>=IF (A70=1, IF((B68+C68+B66+C66)&gt;0,0,1),0)</td>
<td></td>
</tr>
<tr>
<td>76</td>
<td>Serology + ELISA</td>
<td>=IF (A70=1, IF((B68+C68+B67+C67)&gt;0,0,1),0)</td>
<td></td>
</tr>
</tbody>
</table>

Table V: Structure of the summary of outputs module.

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>65</td>
<td>Summary</td>
<td>3rd test</td>
<td>4th test</td>
</tr>
<tr>
<td>66</td>
<td>Is herd+&amp;MRT+?</td>
<td>=IF (AND (H22&gt;0, result 3rdMRT),1,0) [1]</td>
<td>=IF (AND (H22&gt;0, result 4thMRT),1,0) [1]</td>
</tr>
<tr>
<td>67</td>
<td>Is herd+&amp;m-ELISA+?</td>
<td>=IF (AND (H22&gt;0, result 3rd ELISA),1,0) [1]</td>
<td>=IF (AND (H22&gt;0, result 4th ELISA),1,0) [1]</td>
</tr>
<tr>
<td>68</td>
<td>Is herd+&amp;serology+?</td>
<td>=IF (AND (H22&gt;0, result 3rd serology),1,0) [1]</td>
<td>=IF (AND (H22&gt;0, result 4th serology),1,0) [1]</td>
</tr>
<tr>
<td>69</td>
<td>Infected?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>70</td>
<td>=IF (H22=1,1,“”)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>71</td>
<td>Detection of infection</td>
<td>4 tests</td>
<td>5 tests</td>
</tr>
<tr>
<td>72</td>
<td>Serology</td>
<td>=IF (A70=1, IF((B66+C66+D66)&gt;0,0,1),0)</td>
<td></td>
</tr>
<tr>
<td>73</td>
<td>MRT</td>
<td>=IF (A70=1, IF((B67+C67+D67)&gt;0,0,1),0)</td>
<td></td>
</tr>
<tr>
<td>74</td>
<td>ELISA</td>
<td>=IF (A70=1, IF((B68+C68+D68)&gt;0,0,1),0)</td>
<td></td>
</tr>
<tr>
<td>75</td>
<td>Serology + MRT</td>
<td>=IF (A70=1, IF((B68+C68+B66+C66+D66)&gt;0,0,1),0)</td>
<td></td>
</tr>
<tr>
<td>76</td>
<td>Serology + ELISA</td>
<td>=IF (A70=1, IF((B68+C68+B67+C67+D67)&gt;0,0,1),0)</td>
<td></td>
</tr>
</tbody>
</table>

Notes: [1] cells performing simulations of testing subsequent to the first are not shown in the tables.

A70: Infected herd - The aim of this variable is to have a numeric value in the case of extraction of a number of infected animals greater than zero and a text value in the case of no infected animal being extracted. This text value is used by variables B72 through to B76 (results of the testing programme under consideration) [=IF(A70=1;IF((B68+C68)>0;1;0);A70*1)] where the expression A70*1 generates an error message in the case of no infected animal being extracted. This eliminates all iterations with unacceptable values.

Table VI: Structure modifications for the specificity of testing programmes modules*.

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>35</td>
<td>Bulk milk result</td>
<td>FP: Is herd infected according to MRT?</td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>Risk binomial (1, RiskBeta (B25+1, A25-B25+1))</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table VII: Structure modifications for the specificity of testing programmes modules*.

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>45</td>
<td>Bulk milk result</td>
<td>FP: Is herd infected according to milk-ELISA?</td>
<td></td>
</tr>
<tr>
<td>46</td>
<td>Risk binomial (1, RiskBeta (B39+1, A39-B39+1))</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>C</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td><strong>58</strong></td>
<td>RBY - false positive</td>
<td>RBY - true negative</td>
<td>CFT - positive</td>
</tr>
<tr>
<td><strong>59</strong></td>
<td>Risk Binomial (A22, Risk Beta (DS2+1,CS2+DS2+1)) = A22:A59</td>
<td>Risk Beta (A59, 0, Risk Binomial (A59, CS2+DS2+1, CS2+DS2+1):07) = A59</td>
<td></td>
</tr>
</tbody>
</table>

*Only cells containing titles (black characters) and modified formulas (red characters) are shown.*

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>65</strong></td>
<td>Summary:</td>
<td>1st test</td>
<td>2nd test</td>
<td>3rd test</td>
<td>4th test</td>
<td>5th test</td>
</tr>
<tr>
<td><strong>66</strong></td>
<td>Is herd &amp; MRT+?</td>
<td>=IF(A36=1,1,0)</td>
<td>=IF(result 2nd MRT&gt;0,1,0)</td>
<td>=IF(result 3rd MRT&gt;0,1,0)</td>
<td>=IF(result 4th MRT&gt;0,1,0)</td>
<td>=IF(result 5th MRT&gt;0,1,0)</td>
</tr>
<tr>
<td><strong>67</strong></td>
<td>Is herd &amp; m-ELISA+?</td>
<td>=IF(A46=1,1,0)</td>
<td>=IF(result 2nd ELISA&gt;0,1,0)</td>
<td>=IF(result 3rd ELISA&gt;0,1,0)</td>
<td>=IF(result 4th ELISA&gt;0,1,0)</td>
<td>=IF(result 5th ELISA&gt;0,1,0)</td>
</tr>
<tr>
<td><strong>68</strong></td>
<td>Is herd &amp; serology+?</td>
<td>=IF(H59=1,1,0)</td>
<td>=IF(result 2nd serology&gt;0,1,0)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>69</strong></td>
<td>Infected?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>70</strong></td>
<td>=IF(C22&gt;0,0,1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>71</strong></td>
<td>Detection of infection</td>
<td>3 tests</td>
<td>4 tests</td>
<td>5 tests</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>72</strong></td>
<td>Serology</td>
<td>=IF(A70=0,</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>73</strong></td>
<td>MRT</td>
<td>=IF(A70=0,</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>74</strong></td>
<td>ELISA</td>
<td>=IF(A70=0,</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>75</strong></td>
<td>Serology + MRT</td>
<td>=IF(A70=1,</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>76</strong></td>
<td>Serology + ELISA</td>
<td>=IF(A70=1,</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**ELISA** Enzyme-linked immunosorbent assay  
**m-ELISA** milk-ELISA  
**MRT** Milk ring test

<table>
<thead>
<tr>
<th>Programme</th>
<th>Herd sensitivity in case of optimal fertility</th>
<th>Herd sensitivity in case of sub-optimal fertility</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2 serological tests (Q+M)</td>
<td>96.7%</td>
<td>96.7%</td>
</tr>
<tr>
<td>B</td>
<td>3 MRT + one serological test (Q)</td>
<td>94.8%</td>
<td>94.2%</td>
</tr>
<tr>
<td>C</td>
<td>3 MRT (M)</td>
<td>66.0%</td>
<td>63.2%</td>
</tr>
<tr>
<td>D</td>
<td>3 ELISA (M)</td>
<td>98.2%</td>
<td>96.2%</td>
</tr>
<tr>
<td>E</td>
<td>3 ELISA + one serological test (0)</td>
<td>99.6%</td>
<td>99.1%</td>
</tr>
<tr>
<td>F</td>
<td>4 MRT (0)</td>
<td>75.5%</td>
<td>73.1%</td>
</tr>
<tr>
<td>G</td>
<td>5 MRT (0)</td>
<td>81.8%</td>
<td>79.9%</td>
</tr>
<tr>
<td>H</td>
<td>4 ELISA (0)</td>
<td>99.6%</td>
<td>98.4%</td>
</tr>
<tr>
<td>I</td>
<td>5 ELISA (0)</td>
<td>99.9%</td>
<td>99.3%</td>
</tr>
</tbody>
</table>

**Q** Considered by Council Directive 97/12/EC for qualification.  
**M** Considered by Council Directive 97/12/EC for maintenance of qualification.  
**0** Not considered by Council Directive 97/12/EC.