Selection of European Union standard reference sera for use in the serological diagnosis of infectious bovine rhinotracheitis

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Summary: A study, consisting of three trials, was undertaken in order to find standard reference sera for use in serological diagnostic procedures for infectious bovine rhinotracheitis (IBR) in the European Union (EU). A total of forty-nine laboratories participated in the trials, in which panels of positive, weak positive, and negative candidate sera were tested blind by neutralisation, indirect and blocking enzyme-linked immunosorbent assay, indirect immunofluorescence and passive haemagglutination. A serum which scored positive in all tests in all laboratories was selected as a strong positive standard serum. The chosen negative standard serum gave negative results in virtually all tests. A 1/36 dilution of a positive serum was chosen as the weak positive standard serum and only very sensitive techniques were able to score it positive. Three EU reference sera are now available for the standardisation of serological tests for IBR.

KEYWORDS: Bovine herpesvirus 1 - Comparative trial - Diagnosis - European Union - Infectious bovine rhinotracheitis/infectious pustular vulvovaginitis - Serology - Standard reference sera.

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INTRODUCTION

Infectious bovine rhinotracheitis/infectious pustular vulvovaginitis (IBR/IPV) is an economically important disease caused by bovine herpesvirus 1 (BHV1). The disease is associated with respiratory symptoms and genital infection (27). Of the twelve Member States of the European Union (EU), Denmark is free of IBR/IPV following a national eradication campaign conducted between 1984 and 1991; in France, all bull studs used for artificial insemination (AI) are free of BHV1; Switzerland is also free of IBR/IPV (2). In the AI industry, the transmission of BHV1 can occur by using semen collected from acutely- or latently-infected bulls (4, 11, 12, 14, 20, 22, 26). The latency of BHV1 is defined as the persistence of virus in tissues without clinical signs (3, 15), which is usually accompanied by specific antibodies in the serum (sometimes at a low level). Therefore, the identification of latently-infected bulls in AI centres is critically dependent on the use of very sensitive and specific serological tests (10).

The various serological techniques in use include the following:

- virus neutralisation (VN) (5, 6)
- indirect and blocking enzyme-linked immunosorbent assay (ELISA) (16, 18)
- indirect immunofluorescence (IIF) (24, 25)
- passive haemagglutination (PHA) (7, 23).

Comparative studies of the different techniques used for the detection of antibodies against BHV1 indicate the need for international standardisation (13, 17). To date, there has been only limited international standardisation of such techniques (R.J. Lorenz and O.C. Straub, unpublished findings).

A previous comparative study (henceforth referred to as trial I), which was initially performed to evaluate the sensitivity and specificity of the various tests used in several European laboratories, demonstrated that most in vitro serological tests were efficient for the detection of BHV1 antibodies (17). The above study also suggested that a selection of standard reference sera should be made available, particularly for use in the serological testing of bulls for AI purposes.

The present study, undertaken at the request of the European AI Veterinarians Group and consisting of three trials (II, III and IV), describes the preparation and selection of three EU standard reference sera for use in serological tests for BHV1 in cattle.

MATERIALS AND METHODS

Nine laboratories in eight European countries participated in trial II, ten laboratories in nine European countries participated in trial III, and forty-nine laboratories (including regional laboratories) in eleven countries participated in trial IV. Nineteen serum samples and one immunoglobulin G (IgG) preparation were coded at a single laboratory and dispatched to the various participating laboratories. Random code numbers were assigned to each of these laboratories.

These samples were composed as follows:

a) samples 49 (IPIC [taken from a naturally-infected slightly seropositive bull]; ref. 26), 19, 5, 45, 50 and 11 as described by Perrin et al. (17)
b) an IgG preparation from a hyperimmune serum produced in a specific pathogen-free bovine (serum no. 66)

c) serum from an experimentally-infected six-month-old calf collected 35 days after intranasal inoculation (serum no. 67)

d) negative serum from a four-year-old bull from a BHV1-negative population (serum no. 68)

e) 11 negative sera from AI bulls on BHV1-free stud farms (sera nos 69-79).

The sera were variously diluted, coded and dispatched as described below.

**Trial II**

A total of 48 samples was used, as follows:
- three replicates of sample 49 (undiluted IPIC)
- a negative pool of sera 11 and 50
- eight dilutions in the negative pool of each of sera nos 5, 45, 19 and 66 (for each serum, one of the dilutions was replicated three times).

**Trial III**

A total of 30 samples was used, as follows:
- negative sera nos 68-78 inclusive
- serum no. 67 diluted 1/16 in negative serum no. 68
- serum no. 5 diluted 1/18, 1/36 and 1/72 in negative serum no. 68 (three replicates)
- serum no. 45 diluted 1/32, 1/64 and 1/128 in negative serum no. 68 (three replicates).

**Trial IV**

A total of 7 samples was used, as follows:
- negative sera nos 68 and 79
- serum no. 67 diluted 1/16 in negative serum no. 68
- serum no. 5 diluted 1/36 in negative serum no. 68 (two replicates)
- serum no. 45 diluted 1/64 in negative serum no. 68 (two replicates).

Twenty-six laboratories used VN tests for trials II, III and IV. Seven of these laboratories performed the test as described previously for trial I (17), while laboratory 5 employed the same procedure except that a neutralisation time of 24 h was used rather than 1 h. Laboratory 8 used a 2 h neutralisation time for trial II and a 24 h neutralisation time for trials III and IV. The remaining 17 laboratories performed the VN test only for trial IV as follows: seven laboratories used a 24 h neutralisation time, nine used a neutralisation time of 1-2 h, and one laboratory employed a plaque reduction test. Details of some VN test variables are presented in Table I. IIF and PHA tests were performed as described previously (7, 24, 25). One or more ELISA tests were used by each laboratory involved. A non-commercial indirect ELISA was performed as described by four laboratories in trial II (details of this test in three of these laboratories have already been described for trial I; ref. 17), two laboratories in trial III and nine laboratories in trial IV (three of the latter laboratories used new techniques) (Table II). A non-commercial blocking ELISA was performed by three laboratories in trial II (procedures used by two of these laboratories have already been described; ref. 17),
<table>
<thead>
<tr>
<th>Laboratory code no. *</th>
<th>Virus (TCID₅₀)</th>
<th>Neutralisation time (temperature)</th>
<th>Cells</th>
<th>Reading (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Variable **</td>
<td>24 h (37°C)</td>
<td>Calf testis</td>
<td>5</td>
</tr>
<tr>
<td>1</td>
<td>10-30</td>
<td>18 h (37°C)</td>
<td>MDBK</td>
<td>7</td>
</tr>
<tr>
<td>28</td>
<td>90</td>
<td>24 h (4-8°C)</td>
<td>Embryonic bovine lung</td>
<td>4</td>
</tr>
<tr>
<td>29</td>
<td>25</td>
<td>20 h (37°C)</td>
<td>MDBK</td>
<td>3</td>
</tr>
<tr>
<td>30</td>
<td>ND</td>
<td>1.5 h (22°C)</td>
<td>Bovine kidney</td>
<td>7</td>
</tr>
<tr>
<td>31</td>
<td>100</td>
<td>2 h ND</td>
<td>MDBK</td>
<td>3</td>
</tr>
<tr>
<td>32</td>
<td>100</td>
<td>1 h (37°C)</td>
<td>MDBK</td>
<td>8</td>
</tr>
<tr>
<td>33</td>
<td>100</td>
<td>24 h (37°C)</td>
<td>Calf testis</td>
<td>2</td>
</tr>
<tr>
<td>34</td>
<td>100</td>
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<td>MDBK</td>
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</tr>
<tr>
<td>46</td>
<td>100</td>
<td>24 h (37°C)</td>
<td>MDBK</td>
<td>6</td>
</tr>
<tr>
<td>9</td>
<td>100</td>
<td>24 h ND</td>
<td>MDBK</td>
<td>ND</td>
</tr>
<tr>
<td>47</td>
<td>100</td>
<td>2 h (37°C)</td>
<td>EBTR</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>30-300</td>
<td>20-24 h (37°C)</td>
<td>EBTR</td>
<td>4</td>
</tr>
<tr>
<td>44</td>
<td>100</td>
<td>2 h (37°C)</td>
<td>MDBK</td>
<td>3-4</td>
</tr>
<tr>
<td>5</td>
<td>30-300</td>
<td>24 h (37°C)</td>
<td>AUBEK</td>
<td>3</td>
</tr>
<tr>
<td>36</td>
<td>100</td>
<td>24 h (37°C)</td>
<td>AUBEK</td>
<td>ND</td>
</tr>
<tr>
<td>37</td>
<td>100</td>
<td>24 h (37°C)</td>
<td>AUBEK</td>
<td>ND</td>
</tr>
<tr>
<td>38</td>
<td>200 PFU ***</td>
<td>overnight (37°C)</td>
<td>AUBEK</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>1 h (37°C)</td>
<td>MDBK</td>
<td>4</td>
</tr>
<tr>
<td>35</td>
<td>100</td>
<td>2 h (37°C)</td>
<td>GBK</td>
<td>3-4</td>
</tr>
<tr>
<td>41</td>
<td>100</td>
<td>2 h (37°C)</td>
<td>Bovine kidney</td>
<td>3</td>
</tr>
<tr>
<td>8</td>
<td>100</td>
<td>24 h (37°C)</td>
<td>MDBK</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>30-300</td>
<td>1 h (37°C)</td>
<td>FCK</td>
<td>3</td>
</tr>
<tr>
<td>22</td>
<td>100</td>
<td>2 h (37°C)</td>
<td>Calf testis</td>
<td>ND</td>
</tr>
<tr>
<td>27</td>
<td>100</td>
<td>1 h (37°C)</td>
<td>Calf testis</td>
<td>4</td>
</tr>
<tr>
<td>7</td>
<td>100</td>
<td>24 h (37°C)</td>
<td>Calf testis</td>
<td>5</td>
</tr>
</tbody>
</table>

* participating laboratories were randomly allotted anonymous code numbers
** log₁₀ virus dilution series incubated with constant serum dilution of 1/2
*** plaque reduction test
TCID₅₀: 50% tissue culture infective dose
MDBK: Madin Darby bovine kidney
AUBEK: Auburn University bovine embryonic kidney
FCK: foetal calf kidney
EBTR: embryonic bovine trachea
GBK: Georgia bovine kidney
PFU: plaque-forming unit
ND: no data
### Table II

**Non-commercial indirect enzyme-linked immunosorbent assay procedures**

<table>
<thead>
<tr>
<th>Laboratory code no. *</th>
<th>Antigen</th>
<th>Control</th>
<th>Serum dilution (and incubation)</th>
<th>Conjugate (and incubation)</th>
<th>Chromogen (and incubation)</th>
<th>Positive cut-off</th>
</tr>
</thead>
<tbody>
<tr>
<td>9, 46, 49 Partly purified</td>
<td>Control antigen</td>
<td>1/50 (2 h at 22°C)</td>
<td>Anti-bovine HRP (1 h at 22°C)</td>
<td>TMB/H₂O₂</td>
<td>&gt;0.25 = positive</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(10-15 min at RT)</td>
<td>0.15 to 0.24 = doubtful</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.15 = negative</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>Control antigen</td>
<td>1/100</td>
<td>Anti-bovine IgG-HRP</td>
<td>OPD/H₂O₂</td>
<td>Delta OD&gt;0.100</td>
<td></td>
</tr>
<tr>
<td>47</td>
<td>Control antigen</td>
<td>1/50</td>
<td>Anti-IgG-biotin</td>
<td>OPD/H₂O₂</td>
<td>Delta OD&gt;0.100</td>
<td></td>
</tr>
<tr>
<td>7, 17 Partly purified</td>
<td>Control antigen</td>
<td>Undiluted (18h-24 h at 22°C)</td>
<td>Anti-IgG-biotin (1 h at 22°C) + avidin-HRP (30 min at 22°C)</td>
<td>TMB/H₂O₂ (15 min at 22°C)</td>
<td>OD test &gt;3</td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>Crude supernatant trapped by monoclonal antibody</td>
<td>Wells without antigen (overnight at 4°C)</td>
<td>4 monoclonal anti-bovine IgG1/IgG2-HRP (1 h at 37°C)</td>
<td>OPD/H₂O₂ (10 min at RT)</td>
<td>Delta OD&gt;100</td>
<td></td>
</tr>
<tr>
<td>36 Partly purified</td>
<td></td>
<td>1/50</td>
<td>Anti-bovine IgG-HRP</td>
<td></td>
<td>OD&gt;200</td>
<td></td>
</tr>
</tbody>
</table>

* see Table I

HRP: horseradish peroxidase
TMB: tetramethyl benzidine
OD: optical density
OPD: *ortho*-phenylenediamine
IgG: immunoglobulin G
RT: room temperature
<table>
<thead>
<tr>
<th>Laboratory code no. *</th>
<th>Antigen</th>
<th>Serum dilution (and incubation)</th>
<th>Conjugate (and incubation)</th>
<th>Chromogen (and incubation)</th>
<th>Definition of results</th>
</tr>
</thead>
</table>
| 7, 17, 18            | Partly purified virus    | Undiluted (18-24 h at 22°C)     | Anti-BHV1 biotin (1 h at 22°C) + avidin-HRP (30 min at 22°C)                              | TMB/H₂O₂ (labs 7 and 18) (15 min at 22°C)   | >50% blocking = positive  
30% to 49% = doubtful positive  
<30% = negative |
| 3, 42, 43, 44, 45    | Partly purified virus    | Undiluted (18 h at 37°C)        | Anti-BHV1 gB-specific monoclonal-HRP (1 h at 37°C)                                       | ABTS/H₂O₂ (2 h)                            | >50% blocking = positive |
| 4                    | Partly purified virus    | Undiluted (3 h at 37°C)         | Anti-BHV1-HRP (1 h at 37°C)                                                              | ABTS/H₂O₂ (30 min at 22°C)                  | >25% blocking = positive |
| 9                    | Partly purified virus    | Undiluted (18-24 h at 22°C)     | Anti-BHV1 biotin (1.5 h at 22°C) + avidin-HRP (30 min at 22°C)                          | TMB/H₂O₂ (10-15 min at 37°C)               | >50% blocking = positive  
30% to 49% = doubtful  
<30% = negative |

* see Table I

ABTS: 2,2'-azino-di (3-ethylbenz-thiazoline sulfonate)  
BHV: bovine herpesvirus  
HRP: horseradish peroxidase  
TMB: tetramethyl benzidine  
OPD: *ortho*-phenylenediamine  
gB: glucoprotein B
three laboratories in trial III and ten laboratories in trial IV (Table III). Six coded commercial indirect ELISA kits were used by nine laboratories in trial II, eleven laboratories in trial III and forty-six laboratories in trial IV.

RESULTS

Trial II

Serum no. 49 was considered a primary reference serum; this weak positive serum was collected from a naturally-infected bull two weeks after virus had been isolated. As serological tests for detection of BHV1 antibodies should be sensitive enough to detect infection in this bull, dilutions of several sera were compared to select a sample which could be used as a weak positive standard serum.

The simple regression of the optical density values for ELISA tests and VN titres was calculated using the multiplicative model $y = ax^b$ for each serum tested in dilution and for each test used in the different laboratories. Using the regression method, a dilution was calculated for each test serum which would give the same activity in that test as the primary reference serum (serum no. 49: IPIC). The following results were eliminated: non-significant regression and calculated dilution out of the titration curve. The results of each test for each serum are shown in Figure 1.

Two sera (nos 5 and 45), when diluted (1/18 and 1/32 respectively) to correspond with serum no. 49, showed the lowest coefficient of variation and were consequently chosen for use in the next two trials.

Trial III

The main purpose of this trial was to determine which dilution of the sera selected by trial II could be used to estimate the sensitivity of the various techniques.

Results for the eleven negative sera showed 100% specificity for the majority of the tests, the exceptions being three false-positive reactions in the VN test (serum no. 77, titre 1 in laboratories 2 and 9; and serum no. 75, titre 1 in laboratory 9) and one false-positive reaction in commercial indirect ELISA test code 2 (serum no. 68, used for the dilutions of positive sera, in laboratory 8).

The strong positive serum (no. 67) was found positive in all tests.

Two sets of three serial dilutions of positive sera (nos 5 and 45) in negative serum (no. 68) were examined; the results are given in Figure 2. Most of the tests – except the commercial indirect ELISAs and the PHA – found the first two serum dilutions positive. The blocking ELISAs and the IIF gave positive results for the three dilutions for serum no. 5. The non-commercial indirect ELISAs and the IIF gave positive results for the three dilutions of serum no. 45. The blocking ELISA from laboratory 7 found the various dilutions of serum no. 45 doubtful or negative.

Dilutions 1/36 of serum no. 5 and 1/64 of serum no. 45 were chosen for use in the next trial.

Trial IV

In this trial, the number of participating laboratories was extended to widen the basis for making a more appropriate choice between the two candidate weak positive sera,
Geometric means of dilutions corresponding to the primary standard reference serum no. 49 (trial II)
and to examine whether the positive and negative sera were correctly scored by the laboratories.

The results for the two negative sera showed 100% specificity for the majority of the tests, with the exception of six false-positive reactions in the same commercial indirect ELISA (test code 2) with serum no. 68. All tests correctly scored the strong positive serum (no. 67) as positive, except the recently-developed blocking ELISA test used in laboratory 9 and a commercial indirect ELISA (test code 1), which both yielded doubtful results.

In addition, two replicates of serum no. 5 (diluted 1/36) and serum no. 45 (diluted 1/64) were examined. The results varied widely depending on the techniques used. Most of these samples were found positive using the VN tests, the blocking ELISAs and two commercial indirect ELISAs (test codes 3 and 7); the latter test (code 7) and the IIF were the only tests which gave 100% positive results for one or both sera. PHA, commercial indirect ELISAs, and serum no. 5 tested by non-commercial indirect

Fig. 2
Comparison of sensitivity of serological techniques based on two weak positive samples and three dilutions (trial III)
ELISAs all gave negative or a majority of negative results. Results yielded by the commercial indirect ELISA test code 2 could not be interpreted, as this test gave false-positive results for serum no. 68 (which was used for preparing the dilutions). The total of all results for all techniques showed a similar rate of positive or doubtful results for the two sera.

In this trial, no technique – except the IIF – always gave positive results for the two replicates of sera nos 5 and 45; the VN tests and the blocking ELISAs had a detection rate of at least 50% (Fig. 3).

**Comparison of sensitivity of serological techniques based on two candidate reference sera (weak positive) performed by forty-nine laboratories in the European Union**
DISCUSSION

Various serological methods (VN, ELISA, IIF and PHA) are in use to detect antibodies for BHV1, including new tests such as blocking ELISAs (9, 13, 17). For international trade activities, a recommended technique should be able to detect all latently-infected cattle and have a high specificity (1). Therefore, there is a need for international standardisation of antibody assays for BHV1 and for the establishment of international standards (13, 17, 19, 21). The best method of ensuring the reliability of tests is to select a weak positive serum which must be scored positive by all tests.

In trial I (17), a primary reference serum (no. 49) was identified, which originated from a naturally-infected bull whose status had been confirmed by virus isolation (26). Unfortunately, the bull was no longer alive and the amount of serum available was limited.

The aim of trial II was to find the dilution of each candidate serum which corresponded to the primary reference serum (no. 49). The two sera chosen (nos 5 and 45) showed the lowest dispersion of calculated dilutions for all the tests, especially with the VN test. Calculation formulae other than $y = ax^b$ were evaluated and gave similar results (data not shown).

The results of trials III and IV showed that the 24 h incubation VN test, IIF and blocking ELISAs were very sensitive. The three false-positive reactions with a low titre using the VN test in trial II were not very significant. It should be noted that one 24 h incubation test (laboratory 9) can sometimes produce false-positive results (8). Surprisingly, the blocking ELISAs from laboratories 7, 17 and 18 found dilutions 1/32 and 1/64 of serum no. 45 doubtful. Of the commercial ELISAs, test codes 3 and 7 proved to be the most sensitive. Most of the laboratories which used the commercial ELISA test code 2 found the negative serum (no. 68) positive, probably due to some problem with this particular kit. However, it was outside the scope of this study to investigate this problem further. With the indirect ELISAs, there was considerable variation in sensitivity, which probably reflects the differences in test protocols (Table II). For example, in trial IV, laboratory 7 consistently scored the weak positive samples as positive whereas laboratory 9 failed to detect any of the weak positives.

On the basis of the results from trial III, it was decided to choose a dilution twice as high as the dilution corresponding to serum no. 49 (1/36 for serum no. 5, and 1/64 for serum no. 45), as maximum test sensitivity is required for AI purposes.

The Office International des Epizooties (OIE) has recommended that standards for serological tests should preferably consist of strong positive, weak positive, and negative sera. Following trial IV, both serum no. 5 diluted 1/36 and serum no. 45 diluted 1/64 appeared to be suitable for use as international standards, as they contained the same concentration of anti-BHV1 immunoglobulins and exhibited the same activities under the different tests. However, serum no. 5 was finally selected to become the international standard. Serum no. 67 (prepared in the same way as serum no. 45) was selected as the strong positive serum, while serum no. 68 (collected from a bull in a country free of IBR) was chosen as the negative standard serum.

In conclusion, the results of trials II, III and IV indicated that serum no. 67 diluted 1/16 (strong positive), serum no. 5 diluted 1/36 (weak positive) and serum no. 68 (negative) should be used as EU standard reference sera for the serological diagnosis of IBR.
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hemaglutinación pasiva. Un suero que resultó positivo mediante todas las pruebas realizadas en cada laboratorio fue seleccionado como suero de referencia altamente positivo. Un suero que resultó negativo mediante virtualmente todas las pruebas fue seleccionado como suero de referencia negativo. Una dilución al 1/36 de suero positivo, que resultó positiva solamente mediante las técnicas más sensibles, fue seleccionada como suero de referencia ligeramente positivo. Tres sueros de referencia de la Unión Europea son ahora disponibles para la estandarización del diagnóstico serológico de la rinotraqueítis infecciosa bovina.


* * *

REFERENCES


