

The experience of contagious bovine pleuropneumonia ring trials in Botswana

This paper (No. 30102018-00134-EN) has been peer-reviewed, accepted, edited, and corrected by authors. It has not yet been formatted for printing. It will be published in December 2018 in issue 37 (3) of the *Scientific and Technical Review*

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Summary

The Botswana National Veterinary Laboratory (BNVL) has conducted ring trials (proficiency testing) on an annual basis since 2010. Proficiency testing is carried out to evaluate the ability of veterinary laboratories to perform serological complement fixation tests (CFTs) and molecular polymerase chain reaction (PCR) tests for the diagnosis of contagious bovine pleuropneumonia (CBPP). In this paper, the authors discuss the experience gained and the lessons learned in coordinating these ring trials over a period of six years, from 2010 to 2015. The number of participating laboratories increased from five in 2010 to 11 in 2015. Their performance also improved over this period. The proportion of unsatisfactory results decreased from 40% to 10% for serological testing, while questionable results decreased from 60% to 10%. The proportion of unsatisfactory results for the molecular test decreased from 33% to 0%. Systematic errors (i.e. technical errors or imperfect experimental design) were the principal causes of questionable and unsatisfactory results. An analysis of responses from customer satisfaction surveys conducted annually since 2013 provided

valuable information that enabled BNVL to redesign the programme in 2014 and 2015 to improve the overall quality of the proficiency testing programme. Among the changes made were sending freeze-dried sera for CFTs and DNA for PCR instead of sera and liquid cultures.

Keywords

Botswana – Botswana National Veterinary Laboratory – Complement fixation test – Contagious bovine pleuropneumonia – *Mycoplasma mycoides* subspecies *mycoides* – OIE Reference Laboratory – Polymerase chain reaction – Proficiency testing – Ring trials.

Introduction

Contagious bovine pleuropneumonia (CBPP), caused by *Mycoplasma mycoides* subspecies *mycoides* (*Mmm*) (1), is one of the most serious transboundary animal diseases. It has a significant socio-economic impact on trade, supply and demand, food security and nutrition (2, 3). It is manifested clinically by fever, anorexia, a deep cough and dyspnoea (2, 3). This disease continues to be a serious threat to livestock production in the Southern African Development Community (SADC) region, and has been reported from the Democratic Republic of Congo, Angola, and some areas of Namibia and Zambia, among other SADC countries (3, 4).

Early, rapid and accurate detection of the causative agent plays a pivotal role in taking suitable and effective preventive measures against CBPP. National veterinary laboratories also play an important part in the diagnosis and surveillance of the disease. The diagnostic capacity of national veterinary laboratories within the African region for CBPP is gradually improving, but the region has relatively few World Organisation for Animal Health (OIE) Reference Laboratories, when compared to other regions. The Botswana National Veterinary Laboratory (BNVL) is the only OIE Reference Laboratory for CBPP in Africa. Other CBPP OIE/Food and Agriculture Organization of the United Nations (FAO) Reference Laboratories are found outside the continent and include the: Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise (IZS) in Italy; Centre de coopération

internationale en recherche agronomique pour le développement (CIRAD), Unité mixte de recherche (UMR), in France; Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail (ANSES) in Lyon (France); and Laboratório Nacional de Investigação Veterinária (LNIV) in Portugal.

One of the roles of the BNVL as an OIE Reference Laboratory for CBPP is to organise proficiency testing (PT) and inter-laboratory comparison (ILC) schemes. Consequently, the BNVL introduced a ring-trial programme in 2010 to monitor the technical capabilities of laboratories that perform serological and molecular diagnostic tests for CBPP in the SADC region. The BNVL currently provides PT for the complement fixation test (CFT) (3) and polymerase chain reaction (PCR) (5, 6). Participation in PT schemes is one of the requirements of the Quality Assurance programme (ISO/IEC 17025:2005) and is essential for laboratories that seek International Organization for Standardization (ISO) accreditation (7). By participating in such programmes, national veterinary laboratories can validate their techniques through comparison with other laboratories, thus ensuring reproducibility of their test results. PT provides independent feedback on the quality of results, enabling laboratories to monitor and improve their performance over time. Additionally, laboratories can accumulate evidence for the reliability of their test results, thus providing assurance for their clients.

This paper describes how the BNVL organises and conducts its ring trials for CBPP diagnostic tests. The authors discuss in depth the experience gained and lessons learned during six years of coordinating these ring trials (2010–2015).

Materials and methods

Organisation and design of the contagious bovine pleuropneumonia proficiency testing scheme

In 2008, a scientist from the BNVL was trained at the IZS, Italy, in serological and molecular diagnostic techniques for CBPP and the organisation of ring trials, under the auspices of an OIE Twinning

Project between the BNVL and IZS. A plan to organise the ring trials was developed in 2009. A flow diagram depicting the design of the CBPP ring trials is shown in Figure 1. This PT was organised according to the IZS protocol: 'Protocol for evaluation assays among laboratories performing CBPP serological tests' (unpublished); the OIE Guidelines on Laboratory Proficiency Testing (8); and the South African National Accreditation System (SANAS) R80-03: 'Proficiency testing and other comparison programme requirements for testing and medical laboratories and blood transfusion services' (9).

Insert Figure 1 here

Documentation and invitations to participants

The documents needed for the ring trials were compiled and controlled under the BNVL Quality Management System. They were then revised, based on feedback from customer satisfaction surveys. Invitations to participate in PT were sent out at least a month in advance to participating laboratories in the SADC region. The ring trials were also promoted by BNVL staff during training sessions, workshops and conferences. The participants were given a deadline within which to apply. Every year, invitations were extended to additional countries outside the SADC region. The participating laboratories were assigned a numerical code which was communicated in the instruction form to maintain confidentiality of test results.

Sample preparation

Samples for the complement fixation test

In 2010, all stored field sera testing positive by CFT (10) from the 1995 CBPP outbreak in Botswana (11) were again subjected to the CFT (3) to obtain their titres. Samples to be used for the ring trials were selected based on low and medium titres (from 1/10 to 1/320). Zambia Central Veterinary Research Institute provided positive samples for the 2014 and 2015 PT. Negative serum samples were taken from healthy Botswana cattle. Homogeneity and stability tests (adopted from the IZS protocol) were then performed on each sample.

In order to assess homogeneity, each sample was divided into 15 aliquots. Each of the aliquots was run ten times using the same operator, in the same working conditions, using the CFT (3). Stability was assessed by taking five replicates of each sample, subjecting them to thermal stress ($37 \pm 2^\circ\text{C}$) and examining them at different time intervals (t), stated as; $t_1 = 3$ days; $t_2 = 6$ days and $t_3 = 9$ days. The homogeneity and stability results were analysed by the IZS, using Kruskal-Wallis non-parametric analysis of variance and regression analysis, respectively. The samples selected for PT were then sent to the IZS for confirmation, under the ILC framework. Following confirmation, the sera were aliquoted in 0.5-ml aliquots and each was given a unique identification number. Two negative and three positive sera were sent to each participant from 2010 to 2013. In 2014, six negative and four positive serum samples were sent lyophilised (Alpha 2–4 LSC Christ freeze dryer).

Samples for polymerase chain reaction

The PT samples comprised two positive samples and three negative samples. The positive samples were made from the reference strain *Mmm* 57/13, isolated in 1992 during a CBPP outbreak in Italy (12, 13). Of the three negative samples, two were plain contagious caprine pleuropneumonia (CCPP) broth that had not been inoculated with any organism (14). The remaining sample was CCPP broth that had been inoculated with the reference strain, *Mycoplasma mycoides* subspecies *capri* (*Mmc*) 247/1, isolated in 1992 during a CBPP outbreak in Italy (12, 13, 15). The *Mmc* 247/1 is a better sample to determine specificity and to confirm that the PCR test can distinguish *Mmm* from other genetically related pathogens, such as *Mmc*.

The reference strains *Mmm* 57/13 and *Mmc* were cultured in CCPP broth and incubated for four days at $37 \pm 2^\circ\text{C}$, together with uninoculated CCPP broth, then aliquoted into 2-ml tubes. Before dispatch, all the samples were confirmed according to the BNVL PCR procedure (5, 6). In 2011, 2013 and 2014, the samples were sent as broth cultures to participants, but in 2015 the same number of samples were

sent as 20- μ l aliquots of extracted DNA (Wizard[®] Genomic DNA purification kit, Promega).

Distribution of proficiency-testing materials

The CFT and PCR PT samples were packaged and transported via courier to the participating laboratories, according to International Air Transport Association (IATA) guidelines. During transportation, the samples were stored at $4 \pm 2^{\circ}\text{C}$ to prevent deterioration. They were accompanied by a valid export and import permit, instructions for testing and a result reporting form. Participants were requested to acknowledge receipt of the samples.

Analysis of samples and submission of proficiency-testing results

The participants were instructed to test all the samples under routine conditions in their respective laboratories. They were given a deadline of two weeks after receipt of the materials to finish testing and to report their results on the reporting form provided. The results for CFTs were expressed as either negative or positive. In the event of a positive result, the titre level was expressed and converted into values according to standard guidelines. For PCR results, the laboratories had to indicate the presence (positive) or absence (negative) of *Mmm* in the samples.

Evaluation of participants' performance

The data collected were treated as confidential and reserved solely for analysis and evaluation of the results. From 2010 to 2015, the participants' performance was evaluated only on their test results, using the statistical techniques described below. In 2015, participants were also evaluated on their ability to meet deadlines for the submission of their:

- application to participate in the ring trials
- import permit
- acknowledgement of receipt of their samples
- results
- provision of references/procedures.

Analysis of complement fixation test results (IZS protocol)

The performance of the individual laboratories was determined by calculating the z -score value for each sample, using the following formula:

$$z \text{ score} = \frac{x_i - x_a}{NIQ}$$

Where:

x_i = results provided by the i -nth laboratory

x_a = expected value

NIQ = normalised interquartile range = $(Q3 - Q1) \times 0.7413$

The interquartile range was calculated by working out the difference between the third quartile and the first quartile of the results from all laboratories. The z -scores were interpreted to determine the level of performance (satisfactory, questionable, or unsatisfactory) of the individual laboratories. The interpretation scale was as follows:

$|z| \leq 2$ satisfactory

$2 < |z| < 3$ questionable

$|z| \geq 3$ unsatisfactory

When the number of participating laboratories was fewer than five, results were based only on a descriptive analysis, expressing an opinion on the performance of each laboratory for each sample. The results were not analysed by calculating the z -score. The acceptance criterion was that a result must be no more than one titre above or below the expected value.

The presence of systematic errors (RSZ) in the results was calculated on the basis of the sum of z -scores, as follows:

$$RSZ = \frac{\sum_{i=1}^n z_i}{\sqrt{n}}$$

Where:

n = number of samples tested in each laboratory

Z_i = z -score value of the i -nth sample

The RSZ indexes were interpreted as follows:

$|RSZ| \leq 2$ satisfactory

$2 < |RSZ| < 3$ questionable

$|RSZ| \geq 3$ unsatisfactory

Systematic errors are not determined by chance but are introduced by an inaccuracy (i.e. an observation or measurement) inherent in the system (www.merriam-webster.com/dictionary/systematicerror/).

Analysis of polymerase chain reaction results

Polymerase chain reaction results were evaluated based on the presence (positive result) or absence (negative result) of *Mmm*. The overall performance of each laboratory was determined by the percentage of samples that were correctly identified. The acceptance criteria were based on percentage agreement: $\leq 79\%$ was poor (unsatisfactory) and $\geq 80\%$ was good (satisfactory).

Reporting proficiency testing results

A report was prepared containing all results from participants in an anonymous form, so that laboratories could compare their results with those of other participating laboratories. Advisory and informative comments were offered to participants when necessary, to assist in improving their performance.

Customer feedback and satisfaction survey

A customer satisfaction survey was conducted after the 2013, 2014 and 2015 PT schemes to seek feedback (positive or negative) from the participating laboratories on the overall organisation of the ring trials. The feedback was analysed to improve customer service and the management of the PT. Structured questions were developed and sent via email to laboratories that participated in the PT programmes.

Results

The number of laboratories that participated in CFTs ranged from five in 2010 to nine in 2015, while laboratories taking part in PCR ranged from three in 2011 to eight in 2015 (Table I). At least one OIE Reference Laboratory for CBPP (excluding the BNVL) also took part in the ring trials every year to validate the results. The other participants in the ring trials were primarily from the SADC region. Two laboratories from outside the SADC region took part in 2014 and three in 2015.

Insert Table I here

The performance of the laboratories in CBPP CFTs from 2010 to 2015 is presented in Table II. From 2010 to 2015, the number of laboratories obtaining satisfactory results (correct identification in 80% of tests or more) increased from half to all of them. The percentage of unsatisfactory results decreased from 40% to 10%, while questionable results decreased from 60% to 10% over the six-year period.

Insert Table II here

From 2010 to 2012, 50% of the laboratories that participated in CBPP CFTs in the SADC region obtained satisfactory results (correct identification of at least 80% of samples), while from 2013 to 2015 all the laboratories achieved satisfactory results (Table III).

Insert Table III here

Systematic errors were observed every year among participants and these are presented in Table IV. The errors were found most often in Laboratory 4 in 2010, 2011 and 2015, followed by Laboratory 2 in 2013 and 2015.

Insert Table IV here

In 2011, two out of three laboratories that participated in PCR PT obtained satisfactory results. All the laboratories that participated in 2013, 2014 and 2015 obtained satisfactory results (Fig. 2).

Insert Figure 2 here

Discussion

Over all, the development and organisation of the CBPP ring trials has improved since their inception in 2010. Although PT started with only CFTs, PCR was added later. Feedback through customer satisfaction surveys provided valuable information which helped to improve the design of the programme, as shown in Figure 1. Changes were made which included sending freeze-dried sera for CFTs and DNA for PCR. Neighbouring countries supported the ring trials by sharing positive samples that were used in the PT. This is because PT material was difficult to obtain in Botswana, which is free from CBPP.

The growth and strength of CBPP PT increased over the years in which it was conducted. The higher the number of participants, the more statistically valid, confident and reliable PT becomes (16). The performance of laboratories improved considerably over the six-year period, with performance levels changing from 'questionable' or 'unsatisfactory' to 'satisfactory'. This was observed in Laboratories 4 and 8 in particular (Table II).

The improvement in laboratory performance is also demonstrated in Table III; here, laboratories in the SADC region obtained scores of more than 80%. Unsatisfactory results gave participating laboratories a chance to plan corrective action to improve their performance. The BNVL visited Laboratory 4 in 2012 to offer technical support to improve its performance.

In addition, CFT protocol was harmonised and adopted in the 2013 CBPP Regional Scientific Network Workshop, to ensure that all laboratories were testing to an acceptable standard; one that is known to produce accurate and reliable results. In April 2015, five laboratories took part in a two-week training course on CBPP diagnostic tests: CFTs, competitive enzyme-linked immunosorbent assay (cELISA), culture, isolation, and PCR. Finally, PT results are presented annually to participants during the CBPP Regional Scientific Network meetings.

Unsatisfactory and questionable performances could be attributed to the systematic errors observed over the years, as indicated in Table IV. The sources of systematic errors included, among others:

- poor maintenance of instruments
- changes in environment, such as temperature
- human limitations (faulty human observations)
- imperfect methods of observation.

Adequate implementation of the quality management system could reduce such systematic errors. Interestingly, the questionable results of Laboratory 5 in 2015 were not attributable to systematic errors but could be traced back to a delay in sample delivery, due to Customs restrictions at the importing country. The samples were exposed to inappropriate storage conditions (a temperature of 40°C for days) beyond the period (nine days) in which the panel remains stable during the stability test.

Many challenges were encountered over the six-year period of the ring trials. They included the following:

- i)* a shortage of positive samples for use in conducting CFTs and PCR PT, since Botswana is free from CBPP. Even though another country provided some positive samples for PT, they were not enough. The BNVL continues to seek support, in the form of sharing samples, through the CBPP Regional Scientific Network;
- ii)* a low participation rate in the PT. Initially, the number of participating laboratories was as low as five, resulting in too small a sample size for statistical analysis, as indicated in the 2013 customer satisfaction survey. The BNVL promoted PT through workshops,

training courses, seminars and the CBPP scientific network and, by 2015, participation had increased to 11 laboratories;

iii) certain countries prohibited the use of specific couriers and this constrained the shipment of samples to participating laboratories. This was evident in 2012, when one laboratory did not receive the samples at all. To remedy this, the BNVL amended the invitation forms, asking participants to indicate their preferred courier companies;

iv) participating laboratories did not meet deadlines. This presented a challenge in regard to timely analysis of the ring trial results and laboratory feedback.

Analysing responses in the customer satisfaction survey has provided valuable information for future improvements in the design of the programme. Consequently, a customer satisfaction survey will be carried out after each PT distribution so that the programme can be continually updated and enhanced. Looking towards the future, it is planned to enrich the PT by including evaluations of the sensitivity and specificity of the techniques used by each participating laboratory, and to accredit the PT programme, according to reputable international standards (17).

Conclusion

An analysis of the six-year CBPP PT has demonstrated significant improvements in the performance of the participating laboratories. This is evidenced by the reduction in questionable and unsatisfactory results over the period that the ring trials have been conducted. Factors that contributed to an unsatisfactory and/or questionable performance were highlighted and suggestions on how to improve performance were made. The BNVL will continue to conduct PT annually, to ensure continuous improvement in the performance of the national veterinary laboratories in the SADC region.

Acknowledgements

The authors would like to thank: the IZS for analysis of the PT results; Zambia Central Veterinary Research Institute for providing PT samples; and Dr Joseph Hyera (Botswana Vaccine Institute) and the late Dr Mercy Kawonga (BNVL) for editing the manuscript. The Twinning Project between the BNVL and IZS on CBPP diagnostic techniques, which ultimately culminated in the CBPP ring trials, was funded by the OIE, to which the authors are indebted. The planning and operation of the CBPP ring trials was funded by the Government of Botswana through the Director of Veterinary Services, Ministry of Agricultural Development and Food Security. Special thanks are due to field personnel, the Disease Control Unit and all those who helped in many various ways; to all of you, the authors record their gratitude.

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Table I**The number of laboratories that participated in the contagious bovine pleuropneumonia diagnostic proficiency testing, 2010–2015**

| Year | Diagnostic test | No. of laboratories |
|------|-----------------|---------------------|
| 2010 | CFT | 5 |
| 2011 | CFT | 5 |
| | PCR | 3 |
| 2012 | CFT | 4 |
| | PCR | – |
| 2013 | CFT | 6 |
| | PCR | 3 |
| 2014 | CFT | 6 |
| | PCR | 4 |
| 2015 | CFT | 9 |
| | PCR | 8 |

CFT: complement fixation test
PCR: polymerase chain reaction

Table II
Laboratory performance in the contagious bovine pleuropneumonia complement fixation test, 2010–2015

Performance is expressed as a percentage of satisfactory, unsatisfactory and questionable results

| Year | Results | Laboratories | | | | | | | | | |
|------|---------|--------------|------|------|------|------|-----|------|-----|------|--|
| | | 1 | 2 | 3 | 4 | 5 | 7a* | 7b* | 8 | 10 | |
| 2010 | S | 60% | 80% | 80% | 20% | 100% | DNP | DNP | DNP | DNP | |
| | U | 40% | 0 | 0 | 20% | 0 | | | | | |
| | Q | 0 | 20% | 20% | 60% | 0 | | | | | |
| 2011 | S | 100% | 100% | 40% | 60% | 100% | DNP | DNP | DNP | DNP | |
| | U | 0 | 0 | 20% | 0 | 0 | | | | | |
| | Q | 0 | 0 | 40% | 40% | 0 | | | | | |
| 2012 | S | 100% | 100% | 60% | 60% | DNP | DNP | DNP | DNP | DNP | |
| | U | 0 | 0 | 0 | 0 | | | | | | |
| | Q | 0 | 0 | 40% | 40% | | | | | | |
| 2013 | S | 100% | 80% | 80% | 100% | 100% | 60% | DNP | DNP | DNP | |
| | U | 0 | 20% | 20% | 0 | 0 | 40% | | | | |
| 2014 | S | 100% | 100% | DNSR | 100% | 100% | DNP | DNP | 70% | DNP | |
| | U | 0 | 0 | | 0 | 0 | | | 30% | | |
| 2015 | S | 100% | 90% | 100% | 90% | 90% | DNP | 100% | 90% | 100% | |
| | U | 0 | 0 | 0 | 10% | 0 | | 0 | 0 | 0 | |
| | Q | 0 | 10% | 0 | 0 | 10% | | 0 | 10% | 0 | |

DNP: did not participate

DNSR: did not submit results

Q: questionable results

S: satisfactory results

U: unsatisfactory results

From the Southern African Development Community region: 1, 2, 3, 4 and 10

*Laboratories 6 and 9 did not participate in the complement fixation test. Laboratory 7a ended its participation in 2014 so a new participating laboratory (7b) was given its identification number

Table III
Performance of laboratories in the Southern African Development
Community region, 2010–2015

| Year | Number of laboratories | | |
|------|------------------------|-----------------------|--------------------------------|
| | Participating | Scoring more than 80% | Providing satisfactory results |
| 2010 | 4 | 2 | 50% |
| 2011 | 4 | 2 | 50% |
| 2012 | 4 | 2 | 50% |
| 2013 | 4 | 4 | 100% |
| 2014 | 3 | 3 | 100% |
| 2015 | 5 | 5 | 100% |

Table IV
Laboratories in which systematic errors were observed, 2010–2015

| Year | Results | Laboratories | | | | | | | | |
|------|---------|--------------|---|------|---|-----|-----|-----|-----|-----|
| | | 1 | 2 | 3 | 4 | 5 | 7a | 7b | 8 | 10 |
| 2010 | U | ✓ | – | – | ✓ | – | DNP | DNP | DNP | DNP |
| 2011 | U | – | – | ✓ | ✓ | – | DNP | DNP | DNP | DNP |
| 2012 | – | – | – | – | – | DNP | DNP | DNP | DNP | DNP |
| 2013 | Q | – | ✓ | – | – | – | ✓ | DNP | DNP | DNP |
| 2014 | U | – | – | DNSR | – | – | DNP | DNP | ✓ | DNP |
| 2015 | Q | – | ✓ | – | ✓ | – | DNP | DNP | ✓ | – |

DNP: did not participate
 DNSR: did not submit results
 Q: questionable results
 U: unsatisfactory results
 ✓: Systematic error

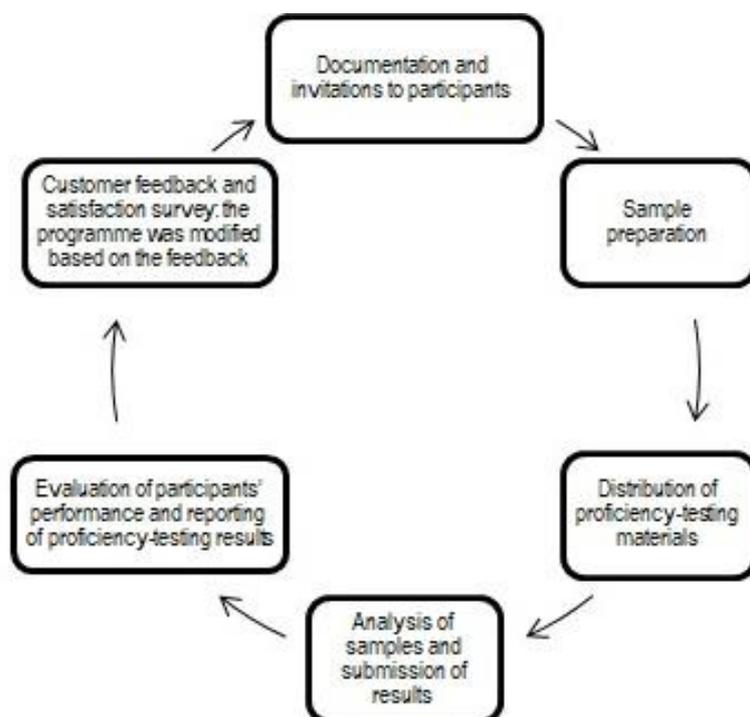


Fig. 1

Flow diagram depicting the design of contagious bovine pleuropneumonia ring trials in Botswana National Veterinary Laboratory

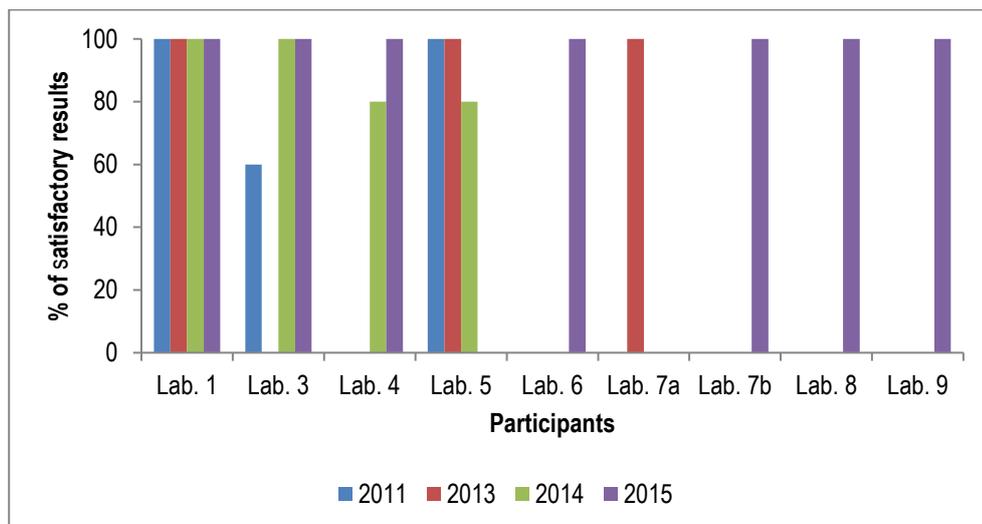


Fig. 2

Overview: percentage of satisfactory results for all participating laboratories in detecting contagious bovine pleuropneumonia using polymerase chain reaction, from 2011 to 2015

Lab.: Laboratory