Diagnostic methods for Q fever in ruminants: contribution to the validation of performances and to their harmonization

Elodie Rousset, T. Lurier, Elsa Jourdain, Richard Thiéry

KEYNOTES

Diagnostic methods for Q fever in ruminants: contribution to the validation of performances and to their harmonization

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Work to improve the quality of diagnostic methods for Q fever is being pursued in France, by the National Reference Laboratory (NRL), together with producers of available commercial kits, analytical laboratories, and with scientific collaborators. Standardization and calibration of methods are a prerequisite for the production of reliable and usable data for a network of laboratories, involved in surveillance programs as well as epidemiological studies, case diagnostics and confirmation or investigations linked to human outbreaks. The role of the NRL is also to ensure that the performance of methods by analytical laboratories, and, their harmonization across a network of laboratories, are properly maintained.

Serological analyzes are carried out in France using three ELISA commercial kits, which use antigens obtained from different strains of *Coxiella burnetii*. Discordant results between kits are observed. Moreover, no reference method exists, and there is no collection of true positive and true negative sera, representative of the diversity of the epidemiological situations encountered for the three main target species (sheep, goat and bovine) bred in French regions. First, to overcome these difficulties, a reference material (RM), provided by the NRL, was included into the manufacturers' quality control. This allows estimating the variability of the measurements around the positivity threshold, which corresponds to the critical zone, and defining calibration criteria for each kit batches. Second, a comparative study was undertaken using a probabilistic modeling approach to better characterize the diagnostic performances of the kits in clinical or epidemiological contexts (PhD in progress). The results are expected to assess the kits' specificities and sensitivities. Based on these characterizations, a common standard serum for all kits, or even a common reference serological antigen, could be developed to be available to kit producers.

Real-time PCR methods, based on commercial kits, were validated in compliance with the U47-600 standard provided by the French normalization body (AFNOR), and, harmonized within the framework of a network of laboratories. Because these methods are used for the etiological diagnosis of abortion to Q fever, a bacterial load threshold was suggested. Then in order to reduce the financial costs associated to quantitative PCR (qPCR), a principle of PCR relating to this clinical interpretation threshold (relative PCR, rPCR) has been proposed. A list of validated 23 qPCR and rPCR methods has thus been established and recommended in France for the clinical diagnosis of laboratories. Adoption assays were performed, in laboratories conditions, to confirm initial performance of a specific method before routine analysis. Instructions on how maintaining this performance were provided, in particular on the basis of a bacterial RM and a control chart. Beyond this global harmonization work, additional studies must also be carried out to consolidate or change the definition of the threshold.

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DIAGNOSTIC METHODS FOR Q FEVER IN RUMINANTS:

CONTRIBUTIONS TO THE VALIDATION OF PERFORMANCES AND TO THEIR HARMONIZATION

MEETING OF THE NATIONAL REFERENCE LABORATORY OF CHLAMYDIOSES, PARATUBERCULOSIS (PTB), BOVINE TUBERCULOSIS (BT) AND Q FEVER (QF)

FLI, GERMANY, WEB CONFERENCES, 2021 21-23

Missions of the French RNL for Q fever

Reference laboratory at:

> national level * (mandated by the French Ministry of Agriculture)

> international level since 2013 (OIE)

Contributions to research projects / reference activities / expertises for:

- ❑ Methods for diagnosis and epidemiology
- ❑ Measures for sanitary management (protection of animal and public health)
- ❑ Investigations linked to clustered human cases (health crisis, risks of exposure)
- ❑ Epidemiology (understanding the infection, contribution to monitoring)

**List of RNL in France at ANSES : https://www.anses.fr/en/content/reference-mandates*

Sanitary situation of Q fever in ruminants in France

First large survey in ten departments

(2012-2015 / 10 labs)

Gache *et al*, Epidemiol. Infect. 2017

No mandatory monitoring in France

(E category within new Animal Health Law in Europe => to prepare)

Data at herd level

Species	Total (episodes)	% Pos	Min-Max (department)
Cattle	3 324	**2,7**	0 – 5.1
Sheep	776	**6,2**	0 – 17.9
Goats	114	**15,8**	0 – 36.4

Q fever abortive episodes

OSCAR

(27 departments)

Species	Total (herds)	% Pos	Min-Max (department)
Cattle	731	**36**	6.4 – 75.5
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***C. burnetii* infection serological survey**

Reference missions on available tests for current Q fever diagnosis

In France, the tests routinely used in veterinary laboratories are

(10 mandated laboratories, 27 OSCAR volunteer departments, 50-80 participating labs in ILPTs)

Serological methods :
Several indirect ELISA commercial kits

PCR methods (DNA extraction + PCR run) :
Commercial kits and homemade methods

Aim = to ensure the quality of the results / the reliability of the methods

- **Standardization + validation** => Defined and maintained performances of a fixed SOP
- **Harmonization** => Comparable results from several methods performed by a network of labs

Diagnostic methods for Q fever in ruminants: contribution to the validation of performances and to their harmonization

1 — Contributions of the QF-RNL to the real-time PCR tests

Validations carried out in collaboration with PCR kits manufacturers

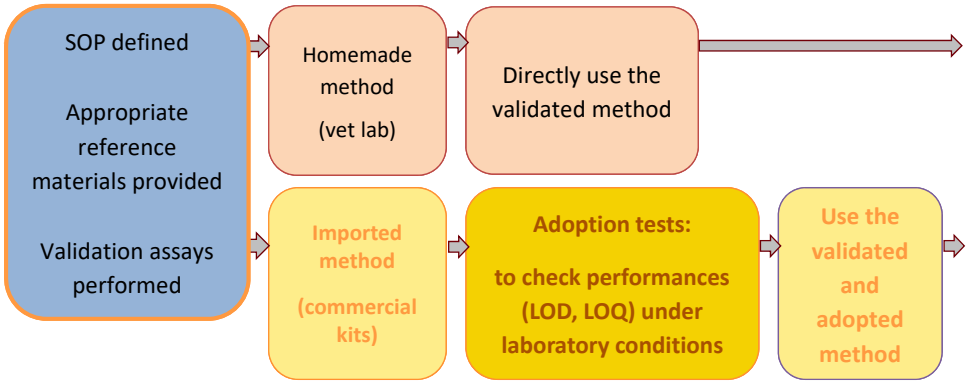
- Priority on methods required for the **diagnosis of abortion series**
- Validation in accordance with:
  - the French ***AFNOR U47-600*** **standard** (first published in 2011)
  - the **QF-NRL requirements** and **conformity criteria**

- **Biological matrices targeted**: vaginal or endocervical mucus and placental cotyledons
- **Two thresholds**: 10^4 bact / mL (individual) and 10^3 bact / mL (pool of 3 animals)
- **Quantification**
  - including a maximum of 10^6 bact / mL and the thresholds (LOQ < 10^3 bact / mL)
  - a 5-point range
  - accuracy maximal limits of ± 0.70 log10 bact / mL on the entire quantification domain

Conformity criteria for assay validation / each Performance characteristic

Diagnostic methods for Q fever in ruminants: contribution to the validation of performances and to their harmonization

Adoption by labs for the implementation of a new or a modified method

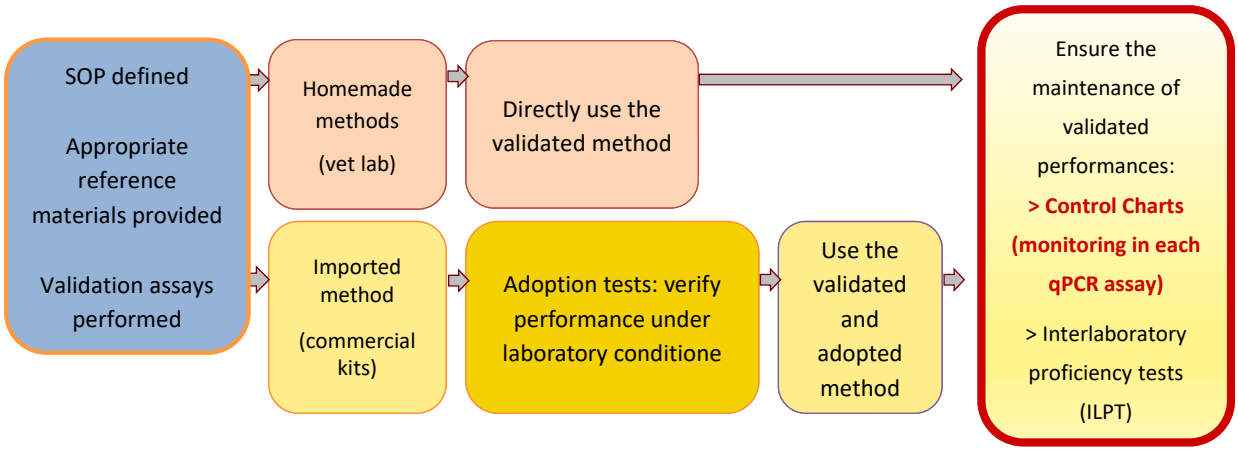


"Routine" analyzes

Rousset *et al*, Euroreference 2012

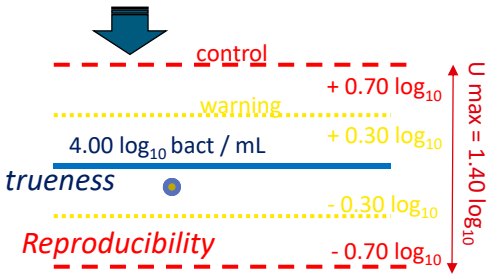
https://pro.anses.fr/euroreference/Documents/ER08-Meth-FievreQAvortEN.pdf

Continuous verification of routine analyzes (internal control chart)



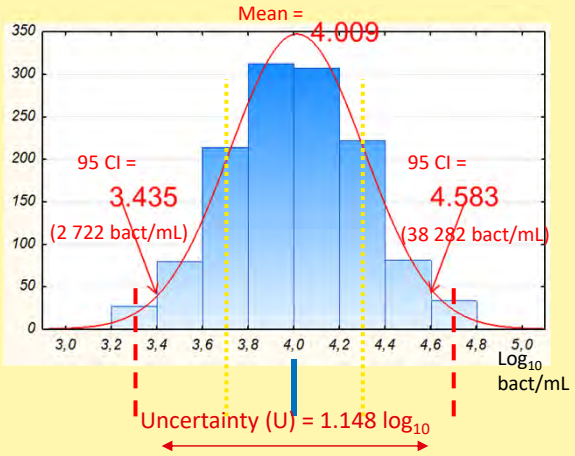
Control chart (CC) data from a laboratory network

Bacterial CC tracer (prepared from the QF-RNL Reference Material) **included in each tested series**



Verification of the value obtained in comparison with the expected value and the maximum authorized limits at 0.70 log_{10}

Distribution of 1,274 tracer data obtained by 10 networked laboratories over 3 years



Inter-laboratory proficiency tests (ILPT) for Q fever PCR in 2018

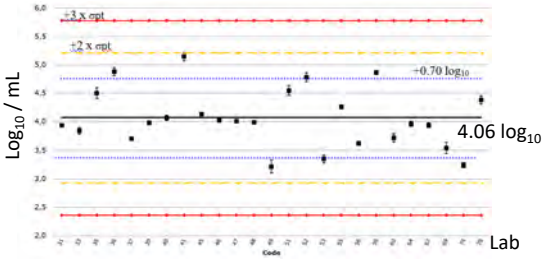
Data obtained by 25 participating laboratories

- Precision and trueness obtained by each lab
- Global mean at 4.06 log_{10}
- Global standard deviation at 0.56 log_{10}

=> **Measurement uncertainty U =** 2 x 0.56= **1,12 log_{10},** in this **ILPT network labs,** for results close to the ""the threshold currently considered to attribute abortions to Q fever" at 4 log_{10} bact / mL (individual)

S4 sample

Results of the measurements in repeatability conditions (three repetitions per test)



Consensus reference quantitative values

Sample ID	Value (in log_{10} bacteria/ml)	Measurement range
S3	3.33	2.85 – 3.81 (σ = 0.48)
S4	4.06	3.50 – 4.61 (σ = 0.56)
S5	5.24	4.66 – 5.82 (σ = 0.58)

Relative (or semi-quantitative) PCR for abortive diagnosis : rPCR

In the rPCR mode, the tracer is calibrated to set at the interpretation threshold

The maximal limits on the control chart are:

+/- 0.70 log10 for the tracer using qPCR

+/- 2.33 Ct for the tracer at interpretation threshold using rPCR

more affordable cost than qPCR

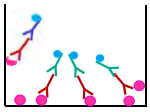
2 — Contributions of the QF-RNL to the serological ELISA tests

Available serological methods

No reference method (no collection of samples, no validation of methods):

- ✓ ELISA and IFI generally shown to be more sensitive than CF
- ✓ IFI less robust than ELISA (operator dependent IFI reading)
- ✓ CF is no longer prescribed by the OIE for international trade

3 semi-quantitative ELISA kits



Indirect ELISA kit	IDEXX	PrioCHECK	ID Screen
Antigen (*C. burnetii* strain)	Nine Mile (reference)	Ovine Cb (French isolate)	Bovine Cb (French isolate)
Conjuguate (HRP) binding	To ruminant IgG	To multi-species IgG (protein G)	
Thresholds set by manufacturers (anti-Cb antibody rates in %OD, Optical Density)	Negatif < 30 OD% 30 ≤ Doubful < 40 Positive ≥ 40	Negatif ≤ 40 OD% 40 < Positive + ≤ 100 100 < + + ≤ 200 200 < + + + ≤ 300 Positive ++++ > 300	Negatif ≤ 40 OD% 40 < Doubful ≤ 50 50 < Positive ≤ 80 Strongly positive > 80

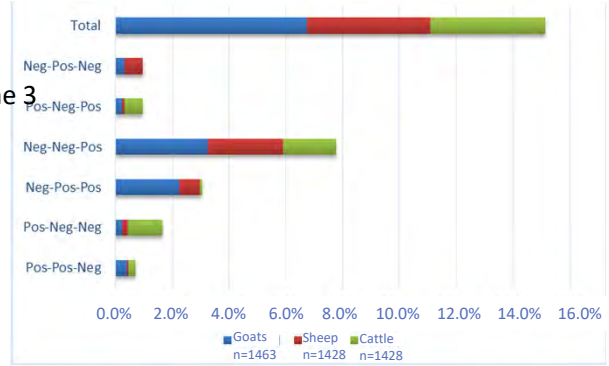
Discordant results between available ELISA tests

Example

4319 sera analyzed under the same conditions with the 3 kits

⇨ 15% of discordant results !

(other than Neg-Neg-Neg and Pos-Pos-Pos)



Performances for harmonization ?

- Difference in specificity ? *Serological Ag involved ?*
- Difference in sensitivity ? *Threshold not set correctly ?*
- Bad reproducibility (precision) and trueness ? *Variability between kit batches ?*

Calibration of kit batches

Reference Material-calibrating (RNL)

Preparation:
2 levels around the threshold of each tested kit

Idexx->1:1 & 1:2
PrioCHECK->1:2 & 1:4
ID Screen->1:4 & 1:8
RM Certificate

Experimental plan

Tested batch :

- ✓ 3 independant assays (1 to 3 operators),
- ✓ 20 repetitions minimum (for each level)

Calculation of variability parameters (RNL file) **:**

- mean (trueness)
- repeatability
- inter-series SD
- reproducibility (limits)
- coefficient of variation

Kit batch certificate

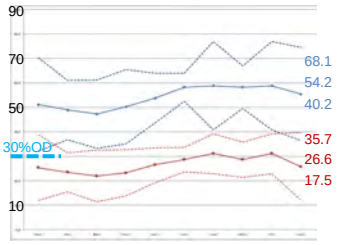
Batch of External Reference Serum (French MRE) : SCE1/2011-12

Lot du MRE (français) utilisé

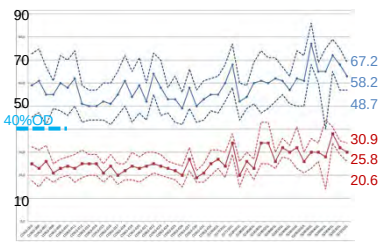
MRE dilution Niveaux du MRE calibrant FQ	Titre Titre	Low limit of 2 Standard Deviation Limite basse de 2 écart-types	High limit of 2 Standard Deviation Limite haute de 2 écart-types	Repeatability Répétabilité (%CV)	Reproducibility CV (%) de fidélité intermédiaire (FI)
1/2	54	43	65	8.3	10.3
1/4	24	18	30	8.4	13.0

Calibration of kit batches

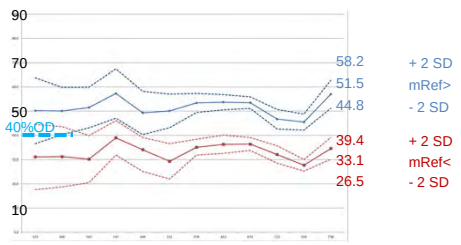
Monitoring of between-batch standardization : data reported on batch certificates (since 2012)



ELISA 1 (27 batches*)
Threshold at 30%OD
RM at 1:1 & 1:2
U = 14.0 & 9.1
CV% = 11.4 & 10.4



ELISA 2 (45 batches)
Threshold at 40%OD
RM at 1:2 & 1:4
U = 9.0 & 5.2
CV% = 7.6 & 6.2

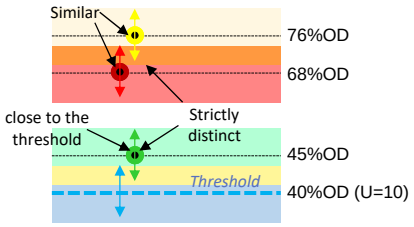


ELISA 3 (12 batches)
Threshold at 40%OD
RM at 1:4 & 1:8
U = 6.7 & 6.3
CV% = 7.2 & 10.8

Define the maximal limits and the expected values

Importance of measurement calibration for semi-quantitative ELISA

- **Acceptance criteria of precision (limits) and trueness (expected value) at the positivity threshold**
  - data to help with batch acceptance by the laboratory user
  - a single control chart monitored for successive batches



- **A standardization over time for each ELISA kit =** Threshold and uncertainty at threshold are controlled
  - Similar and strictly distinct results / **Comparisons or evolutions (statistical differences)**
  - the "Doubtful" in the diagnosis / **"Positive or negative close to the threshold"**

Conclusions

Take home messages

- The **tools** (reference materials, standardization, validations, adoptions, bilateral tests, control charts, inter-laboratory tests) contribute to the **reliability of methods**
  - within each laboratory's environment
  - within a network of laboratories

  *Thus, to determine whether the results could be gathered at national level and used for **infection control, epidemiological investigation or monitoring** (e.g. for new AHL)*
- As NRL, we encourage the presentation of **results with their level of uncertainty**, inherent in any measurement method. This is also a performance characteristic.
- The exchanges* with diagnostic laboratories and kit producers provide means for **proactive improvement**

**Rousset et al, Euroreference 2017: https://euroreference.anses.fr/sites/default/files/17_12_ED_ER_03-1_ROUSSET.PDF*

Work in progress

PCR (real time) :

Progress has been rapid

Reference Material
- ☒ validation (bacteria and gDNA)
- ☒ clinical treshold *(in test)*

Validation (AFNOR standard)
- ☒ methods based on kits (list)
- ☐☒ *other matrices (in progress)*

Vigilance on performances
- ☒ adoptions
- ☒ control charts (qPCRq et rPCR)
- ☒ ILPT (since 2017)

ELISA (indirect) :

A delay in standardization

Reference Material
- ☒ for batch calibration (around set thresholds)
- ☐ at detectability (threshold harmonization)

Validation (scientific publications)
- ☐ comparative evaluation in progress
- ☐ towards reference tools ?

Vigilance on performances
- ☒ bilateral tests (using qualified ILPT panels)
- ☐☒ *batches std (control chart to improve)*
- ☒ ILPT (since >30 years, ELISA since 2001)

Thanks for your attention

Diagnostic methods for Q fever in ruminants: contribution to the validation of performances and to their harmonization

Available Q fever diagnostic methods and objectives of application

Table 1. *Test methods available for the diagnosis of Q fever and their purpose*

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Agent identification						
PCR	+++	n/a	+++	+++	++	+[1]
Culture	+	n/a	+	–	+	–
Staining	+	n/a	+	+	+	–
Genotyping	n/a	n/a	n/a	n/a	++	n/a
Detection of immune response						
ELISA	+++	n/a	+++	++	+++	+++
IFA	++	n/a	++	++	++	++
CFT	–	n/a	–	++	+	+

Key: +++ = recommended method; ++ = suitable method; + = may be used in some situations, but cost, reliability, or other factors severely limits its application; – = not appropriate for this purpose; n/a = not applicable.

From the Chapter "Q fever" of the OIE manual https://www.oie.int/en/standard-setting/terrestrial-manual/access-online/ (Manual of Diagnostic Tests and Vaccines for Terrestrial Animals)

Diagnosis is realized at group level.

(expert reports: French ACERSA 2007, EU EFSA 2010)

No test prescribed for individual diagnosis

In France, official purposes are :

- **Differential diagnosis of abortions *(OSCAR)***
- **Investigations linked to human clusters *(State Note)***
- **Transversal epidemiological survey**

For other purposes, we have to:

-define methods to be used, sampling and results interpretations (sheme for free status, movement and introduction, trade)

-develop other tests (early test, DIVA / vaccine).

Diagnostic methods for Q fever in ruminants: contribution to the validation of performances and to their harmonization

Summary of main diagnostic schemes in France

Purpose	Samples	Targeted animals (Unit sample = herd or kidding group)	Used tests	Basis for interpretation	References
Abortive diagnosis	2 vaginal swabs, 6 sera from cattle 2 vaginal swabs, 10 sera from sheep or goats	max of females having aborted (for < 8 days -> PCR)	qPCR or rPCR ELISA	Clinical threshold (10e4 or 10e3 bact/swab if individual or pool of 3) If one PCR-results is positive, check a 50% seroprevalence	ACERSA (2007) NS DGAL (2010-8262) EFSA (2010) OSCAR (2017->)
Investigation of shedding herds *(clustered human cases)*	20 sera (stratified by 3 ages classes) 15 vaginal swabs *± Environmental samples **	max of females having kidding or aborted for < 1 month (10 primiparous and 5 females 2 to 4 years old, 5 over 4 years old) max of females having kidding or aborted for < 1 month (10 primiparous and 5 multiparous if possible) *a dust sample on a cloth per building and per group of female having kidding during the exposure period*	ELISA qPCR *qPCR*	Analyze sera first Semi- and quantitative data If one ELISA-result is positive (threshold at 10e4) per herd *or if one dust result is positive* *Threshold at 10e4 bact/swab* *Analyze dust first* *Quantitative data*	NS DGAL (2011-8124) *in revision*

**No threshold in terms of transmission risk has yet been established (the use of dust as a risk indicator is at the research stage).*

Sanitary situation of Q fever in ruminants in France, *rapid overview*

No monitoring in France *(cat E within new AHL: in preparation)*

First large survey in ten departments (2012-2015 / 10 labs)

Species	Total (episodes)	% Pos	Min-Max (department)
Cattle	3 324	**2,7**	0 – 5.1
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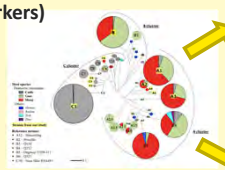
***C. burnetii* infection serological survey**

Gache *et al*, Epidemiol. Infect. 2017

Database of > 300 *C. burnetii* genotypes

(samples from abortions / 2006-2015 / 9 labs)

3 main MLVA genogroups (17 markers)



Similiarities in strains from sheep **(A)** and goats **(B)**

Distinct group with strains froms cattle (NM in this C group)

Joulié *et al*, Infect Genet Evol. 2017