Serogroups and genotypes of *Leptospira* spp. strains from bovine aborted fetuses

Delooz Laurent[1,2], Czaplicki Guy[1], Gregoire Fabien[1], Dal Pozzo Fabiana[2], Pez Floriane[3], Kodjo Angeli[4], Saegerman Claude[2]

[1] Regional Association for Animal Registration and Health (ARSIA) asbl, 5590 Ciney, Belgium

[2] Research Unit of Epidemiology and Risk Analysis applied to veterinary science (UREAR-ULg), Fundamental and Applied Research for Animals & Health (FARAH) Center, Faculty of Veterinary Medicine, University of Liege, 4000 Liege, Belgium

[3] BioSellal, 69007 Lyon, France

[4] Laboratoire des Leptospires, VetAgro Sup, Campus Vétérinaire de Lyon, 69280 Marcy-l'Etoile, France

ABSTRACT

Leptospirosis is a global disease of animals, with potential major economic impact on livestock industry and important zoonotic capacities. The disease represents a major challenge in the developing countries as humans and animals frequently live in close association. The serovar Hardjo of *Leptospira* whose primary host is cattle has been studied extensively, but few data exist on other current circulating or emerging serotypes. To better understand the disease in cattle and how to prevent and/or control it, it is necessary to identify the genotype and the serotype of circulating *Leptospira*. This paper presents results of several investigations performed on a historical Belgian collection of congenital jaundice in bovine aborted fetuses coming from the leptospirosis emerging episode of 2014 (Delooz *et al.*, 2015). The results revealed that *L.* Grippotyphosa and *L.* Australis were the most prevalent serogroups with

respectively 17/42 and 13/42 positive MAT during this emerging event associated with the same clinical pattern. The study also confirms that congenital jaundice is associated with *L. kirscheneri* and *L. interrogans* and provides the genotyping of DNA obtained from these two species.

Keywords: Abortion, Cattle, Icteric, Jaundice, Genotyping, *Leptospira*, Leptospirosis, Belgium

INTRODUCTION

In Belgium, a national surveillance program based on the compulsory reporting of abortions and subsequent analyses on their products reached several objectives including official surveillance of bovine brucellosis but also the monitoring of other bovine abortive diseases. Some emerging or re-emerging pathogens could be identified, including Bluetongue virus serotype 8, *Brucella abortus* and more recently Schmallenberg virus.

Since July 2014, the Belgian Walloon region faced an unexpected situation with a drastic increase of congenital jaundice in bovine aborted fetuses (Delooz *et al.*, 2015). During the last six years, abortions associated with jaundice had been notified but the monthly incidence of cases remained stable. From July to December 2014, an increase of bovine aborted fetuses with jaundice was reported by ARSIA pathologists, with a significantly higher incidence than previous months and years. The standardized panel of analyses designed to extend the diagnosis did not allow identifying the etiology. After additional analyzes, high levels of antibodies against *Leptospira* serogroups Australis and Grippotyphosa were found in cows after abortion of icteric fetuses. Serology performed during the emergence identified serogroups without providing information on the genotype of the involved pathogenic *Leptospira* strains. A leptospiral infection was consequently hypothezised (Delooz *et al.*, 2015).

Leptospirosis is a transmissible disease of animals and humans caused by the spirochete *Leptospira*. All the pathogenic leptospires were formerly classified as members of the species *Leptospira interrogans*. However, the genus has been reorganised and pathogenic *Leptospira* are now classified in 23 species (Adler, 2010; Levett, 2001; Morey *et al*., 2006) from which more than 300 distinct serovars included within 24 serogroups are distinguished (Levett, 2015). Laboratory diagnosis of leptospirosis can be complex and involves tests designed to detect specific antibodies against *Leptospira*, as well as for direct isolation of leptospires, antigens detection and detection of *Leptospira* nucleic acid in animal tissues or body fluids (OIE, 2014).

In cattle, the seroprevalence of the serogroup Sejroe is one of the most studied and varies widely from one country to another with 33% in France (Ayral *et al*., 2014), 30% in the Netherlands (Hartman *et al*., 1989) and up to 83.59% in Ireland (Ryan *et al*., 2012). In addition, antibodies against *Leptospira* serovar Hardjo were found in tank milk of 9.2% bovine dairy herds, with a higher incidence in the southern part of the country (Dom *et al*., 1991). In France, microscopic agglutination test (MAT) performed on samples collected in 394 cattle herds allowed to determine the distribution of the following three predominant circulating serogroups (Australis, Sejroe, and Grippotyphosa) (Ayral *et al*., 2014). In this part of Europe, leptospirosis is prevalent and may be responsible for pathological events in human (Mori *et al*., 2014) and animal health (Delooz *et al*., 2015).

Leptospirosis is a known cause of abortions and infection can be accompanied by a wide variety of clinical signs. However, fetal jaundice was not observed during experimental infections (Ellis and Michma, 1977; Ellis *et al*., 1986; Smith *et al*., 1997). On the contrary based on the recent field observations, the hypothesis of the association of bovine fetal jaundice with leptospirosis was formulated (Delooz *et al*., 2015). Currently, little epidemiological information exists concerning the different serovars of *Leptospira* circulating among cattle in Belgium. While the manifestations of the disease can be very different depending on the strain and the

animal species, it is important to identify the pathogenic strain in order to tackle the best prevention and control measures and thereby, prevent potential transmission to humans (Evangelista and Coburn, 2010).

The aim of this work was to characterize the *Leptospira* infection detected in bovine abortion cases associated with fetal jaundice which occurred in Southern Belgium in 2014.

MATERIAL AND METHODS

Study desing

In the context of the Belgian passive surveillance program for bovine brucellosis, a total of 42 bovine abortion cases collected from October 2011 to December 2014 were included in this study. They originated from 39 cattle farms distributed among the 5 Walloon provinces. They were included in the study according to the diagnosis of congenital jaundice (N = 41) or the PCR positivity against pathogenic *Leptospira* (N = 1).

Information issued from the anamnesis, such as sampling date, herd identification number, cattle breed, month of pregnancy and number of parity were encoded in the laboratory information management system (LIMS) or in an Access® database. None of these herds applied vaccination against *Leptospira* species and, moreover, no *Leptospira* vaccine has a marketing authorization in Belgium. The geographical localization of each case of abortion was possible using the Lambert coordinates and the Belgian cattle identification and movement traceability system (SANITRACE).

Laboratory analyses

A standardized panel of analyses was first applied to perform the laboratory diagnosis of bovine abortion on submitted fetuses. Direct and/or indirect detection of pathogens was performed, including bacteria (*Brucella* spp., *Campylobacter* spp., *Coxiella burnetii*,

Leptospira borgpetersenii and *interrogans* serovar Hardjo, *Listeria monocytogenes*, *Neospora caninum*, *Salmonella* Dublin), viruses (bluetongue virus serotype 8 (BTV-8), bovine herpesvirus 1 (BoHV-1), bovine herpesvirus 4 (BoHV-4), bovine viral diarrhea virus (BVDV), Schmallenberg virus), several mycotic agents, and many other opportunistic bacteria (**Table I**).

Microscopic agglutination tests

MAT was performed on the 42 maternal sera sampled on the aborted cows at the time of abortion using twenty-four serovars representing 14 serogroups of pathogenic *Leptospira* species: Icterohaemorrhagiae, Copenhageni, Australis, Bratislava, Munchen, Autumnalis, Bim, Castellonis, Bataviae, Canicola, Hebdomadis, Panama, Mangus, Pomona, Mozdok, Pyrogenes, Sejroe, Saxkoebing, Hardjo, Wolffi, Tarassovi, Cynopteri, Vanderhoedoni, and Grippotyphosa (**Table II**). According to observations recorded by Chappel and collaborators in 2004, titers of 160 or higher were defined as positive agglutination reactions for ruminants. The end-point was the highest dilution of serum in which 50% agglutination still occured.

Pathogenic *Leptospira* DNA detection (real-time PCR).

During the necropsy, a spleen, kidney, liver and placenta fragment were sampled on each abortion cases, pooled and stored at -20°C. PCR analysis was performed on 26 pools of organs sampled from icteric fetuses, retrieved in the historical abortion samples collection described in the study design (not available for 16 other fetuses). DNA extraction was performed using KingFisher ™ Flex 96 Magnetic Particle Processors (Thermo Scientific ™, UK) and LSI MagVet ™ Universal Isolation Kit (Life Technologies, UK) and was followed by pathogenic *Leptospira* DNA detection using a commercial PCR test (TaqVet ™ PathoLept ™, Thermofisher, France) on organ pool according to the manufacturer's instructions (Levett *et al*., 2005).

The PCR reactions were performed with a Stratagene Mx3500P (Agilent Technologies, USA). According to the manufacturer's instructions, a sample was considered positive with a threshold cycle (= Ct) value lower than 46.

***Leptospira* genotyping method by multilocus sequence typing (MLST)** by Next-Generation Sequencing (NGS)

Among 10 randomly selected samples (i.e. PCR real-time positive group), genotyping was realized in the Biosellal laboratory of Lyon (France) according to the consensus MLST scheme developed by Boonsilp and collaborators (2013). Obtained PCR amplicons were purified using 1.8X Agencourt AMPure XP beads (Beckman Coulter). Qubit fluorometer 2.0 (Life technologies) was used to quantitate and normalize amplicon concentrations, accounting for the different amplicon fragment lengths; this was followed by equimolar pooling of all MLST loci for each sample.

Preparation of Illumina libraries was performed with 1 ng of pooled amplicons according to the Nextera XT protocol (Version January 2015) and libraries were sequenced using the MiSeq Personal Sequencer (Illumina). Bio-Informatic analyses were performed using CLC Genomics Workbench (CLC Bio, Qiagen, Aarhus, Denmark).

The combination of the sequences of these seven loci was compared with a public database, the *Leptospira* spp. MLST Databases. This publication made use of the *Leptospira* MLST website (http://pubmlst.org/leptospira/) developed by Keith Jolley and sited at the University of Oxford (Jolley and Maiden, 2010). This is located at the Imperial College of London and was funded by the Wellcome Trust using Boonsilp and collaborators (2013) protocols (MLST scheme 1) to determine the species of *Leptospira* and serogroup. Briefly, each sequenced MLST locus for one sample was assigned to allele numbers and combined into an allelic profile which is then assigned to a unique sequence type (ST).

RESULTS

Among the 42 abortion cases, using the standardized panel of analyses (**Table I**), it was possible to identify one pathogen in 11 cases, *Anaplasma phagocytophilum* (1), *Coxiella burnetii* (2), *Neospora caninum* (1) including 7 opportunistic bacteria such as *Escherichia coli* (5), *Hafnia alvei* (1), and *Lactococcus lactis* (1). Only one maternal serum was seropositive for the *Leptospira* serovar Hardjo Elisa but the MAT revealed negative results for all the tested serogroups including for serovar Hardjo with low titers of 40.

Microscopic agglutination tests

Among the 42 samples of maternal serum, 29 had a positive result with respect to at least one serogroup (**Table III**). Among the positive samples, agglutination was observed against an average of 2 and a maximum of 5 serogroups per sample. A titer of ≥ 160 was used to define a seropositivity reaction, no positive results have been observed for the following 5 serogroups as Ballum, Bataviae, Canicola, Pomona and Tarassovi. The results revealed that *Leptospira* serogroups Grippotyphosa and Australis were the most prevalent with respectivly 17/42 and 13/42 positive MAT (**Table III**).

Leptospira interrogans spp. (RT-PCR)

Of the 26 organ samples analysed, DNA of pathogenic *Leptospira* was detected in 21 cases. Among the 21 PCR positive cases, 5 sera were negative by MAT against the 24 serovars (14 serogroups) tested.

Concerning the 5 PCR negative cases, a positive MAT was observed in 4 samples. For only one sample, the PCR and the MAT were negative. The results of serology (MAT) and molecular detection (real-time PCR) tests are summarized in **Table III**.

Leptospira MLST genotyping

Among the 10 samples, 2 were successfully amplified and sequenced for all the 7 loci. For these two samples, different sequence types (ST) were obtained, the ST number 110 profile for sample CI-14-061536-002 and the ST number 24 profile for sample CI-12-000889-002 (**Table III**). In the *Leptospira* MLST database, ST number 110 profile corresponds to *Leptospira kirschneri* species and Grippotyphosa serogroup (**Table III**), whereas the ST number 24 profile corresponds to *Leptospira interrogans* species and Australis serogroup (**Table III**).

DISCUSSION

MAT and PCR results support the hypothesis that the jaundice observed in fetuses was due to leptospiral infection. Furthermore, no other cause of abortion was identified despite the wide range of analyzes.

The MAT is the serological reference test, particularly appropriate for carrying out epidemiological studies, since it can be applied to sera from any animal species, and because the range of antigens utilized can be expanded or decreased as required (Levett, 2004). Most cases of leptospirosis are diagnosed by serology and antibodies are detectable in the blood approximately 5 to 7 days after the onset of clinical signs. In our conditions, the sampling was performed at the time of abortion but the presence of jaundice indicated an earlier infection that could explain the relative high titer in MAT observed.

Australis and Grippotyphosa are identified as the two predominant serogroups in this study. These results are consistent with the findings of two other recent studies, in Germany concerning dogs (Mayer-Scholl *et al*., 2013) and in France concerning dogs and cattle (Ayral *et al*., 2014). In France, the two predominant infecting serogroups involved in clinical bovine

and canine leptospirosis from 2008 to 2011 are also Australis and Grippotyphosa for the two species.

On average, sera show a seropositive reaction to two serogroups with a maximum of five. Indeed, MAT is a complex test to control, perform, and interpret. It is a serogroup-specific assay but interpretation of the MAT is complicated by the high degree of cross-reaction that occurs between different serogroups, especially in acute-phase samples. This “paradoxical” reaction, in which the highest titers are detected to a serogroup unrelated to the infecting one, are also common and studied (Blanco *et al*., 2016; Lin *et al*., 1997). The broad cross-reactivity in the acute phase is followed by relative serogroup specificity in convalescent-phase samples (Levett, 2001). Than, an average of two serogroups per sample seems relatively high compared to other studies where only one serovar is highlighted. But this observation argues for paradoxical reaction due to IgM during acute infection. Unfortunatly, paired sera are not available to confirm the infected serogroup with certainty. Moreover, a positive result does not identify with certainty the cause of abortions and it is impossible to date the infection, the reaction kinetics with respect to different serovars may vary (Levett, 2001).

In order to ensure the involvement of bacteria in the abortive process, PCR were performed and bacterial DNA was detected in the great majority of cases. Following these PCR analyzes, different profiles combining PCR and MAT are observed. These profiles may depend on the delay between the infection and the abortion, the immunity of the infected animals, the type of serogroups concerned or the laboratory assays.

In total, in the great majority of cases, both analyzes provide a positive response and support the diagnosis of leptospirosis.

Because of the difficulties associated with serological identification of leptospiral isolates, there has been great interest in molecular methods for identification and subtyping (Terpstra, 1992; Herrmann, 1993). The reclassification of leptospires on genotypic grounds is

taxonomically correct and provides a strong foundation for future classifications. Genotyping of two species of *Leptospira* is a key point that allows knowing with certainty the infecting species. *Leptospira interrogans* and *Leptospira kirschneri* were genotyped and have a positive response to serogroup Australis and Grippotyphosa respectively. These results are consistent with *Leptospira* MLST database.

During this episode in 2014, many questions arise about the almost simultaneous distribution on a broad territory of a disease that does not have the dissemination power of an arbovirus. The disease is maintained in nature by chronic infection of reservoir hosts. The most important reservoir hosts are small mammals, which may transfer infection to domestic farm animals, dogs, and humans. Different rodent species may act as reservoir of the serogroups highlighted in this study (Levett, 2001). Distinct variations in reservoir hosts and the serovars they carry occur throughout the world (Hartskeerl and Terpstra, 1996). Currently, the source of infection remains unknown and therefore complicates the usefulness of implementation of preventive measures. From the affected farms, only one case of bovine aborted fetus was identified in 95% of the farms with a maximum of 3 cases in one farm (Delooz *et al*., 2015). That allows hypothesizing that infection does not spread to the entire herd by other cattle that therefore do not appear to be potential maintenance hosts.

The spectrum of clinical signs is extremely broad. Formerly it was considered that distinct clinical syndromes were associated with specific serogroups. However, this hypothesis was questioned by some authors and following studies refuted this hypothesis (Levett, 2001). Grippotyphosa and Australis are the two main serogroups revealed during this emerging event associated with the same clinical pattern, which joins the idea that clinical syndromes were not associated with specific serogroups. However, congenital jaundice from aborted fetuses coming from clinically healthy cows is a new clinical sign to add to those caused by pathogenic *Leptospira*.

The results of this study indicated that Sejroe serogroup was rarely diagnosed and that methods of the diagnosis of leptospirosis must be adapted for a better surveillance and control. This finding suggests that the available bovine vaccine targeting this serogroup is capable of preventing a minority of the clinical cases. Nevertheless, additional serogroups, such as Grippotyphosa and Australis should be included in the vaccine to eliminate most *Leptospira*-related diseases in cattle.

CONCLUSION

Jaundice was a known clinical sign of leptospirosis but, to our kowledge, had never been diagnosed in bovine aborted fetuses coming from clinically healthy cows in the literature. This work allows the association of two pathogenic *Leptospira* species (*L. interrogans* or *L. kirschneri*) to congenital jaundice in bovine aborted fetuses. This new clinical sign should be added to the clinical picture of bovine leptospirosal abortion.

Leptospira are often difficult to isolate from infected cattle and therefore diagnosis usually depends on the detection of specific antibodies. This work showed a feasible method of direct diagnostic approach under field conditions where the veterinary practitioner performs samples in cattle farms.

Finally, despite that the sources of infection during the emergence remain unknow, this study provided useful information in the knowledge of bovine leptospirosis in south part of Belgium. It seems necessary to be prepared to tackle appropriate prevent and control measures and to further explores the epidemiology of this disease in this region, especially in wild life.

AKNOWLEDGEMENT

We thank Magnée D. (Thermofisher, UK) to provide PCR kits. We thank the Federal Agency for the Safety of the Food Chain (FAFSC) to support the costs of the basic protocol corresponding to the standardized panel of analyses. The authors gratefully acknowledge their veterinary colleagues from ARSIA, i.e. Christian Quinet (serology), Marc Saulmont and Thierry Petitjean (autopsy and microbiology) for their technical assistance.

REFERENCES

Adler, B., 2010: *Leptospira* and Leptospirosis. *Current Topics in Microbiology and Immunology* 387: 1–293.

Ayral, F. C., D. J. Bicout, H. Pereira, M. Artois, and A. Kodjo, 2014: Distribution of *Leptospira* serogroups in cattle herds and dogs in France. *The American journal of tropical medicine and hygiene* 91, 756-759.

Blanco, R. M., L. F. dos Santos, R. L. Galloway, and E. C. Romero, 2016: Is the microagglutination test (MAT) good for predicting the infecting serogroup for leptospirosis in Brazil? *Comparative immunology, microbiology and infectious diseases* 44, 34-36.

Boonsilp, S., Thaipadungpanit, J., Amornchai, P., Wuthiekanun, V., Bailey, M.S., Holden, M.T., Zhang, C., Jiang, X., Koizumi, N., Taylor, K., Galloway, R., Hoffmaster, A.R., Craig, S., Smythe, L.D., Hartskeerl, R.A., Day, N.P., Chantratita, N., Feil, E.J., Aanensen, D.M., Spratt, B.G., and S.J. Peacock, 2013: A single multilocus sequence typing (MLST) scheme for seven pathogenic *Leptospira* species. PLoS Negl Trop Dis. 7(1), e1954.

Chappel, R. J., M. Goris, M. F. Palmer, and R. A. Hartskeerl, 2004: Impact of proficiency testing on results of the microscopic agglutination test for diagnosis of leptospirosis. *Journal of clinical microbiology* 42, 5484-5488.

296 Delooz, L., M. Mori, T. Petitjean, J. Evrard, G. Czaplicki, and C. Saegerman, 2015: Congenital
297 jaundice in bovine aborted foetuses: an emerging syndrome in southern Belgium.
298 *Transboundary and emerging diseases* 62, 124-126.
299 Dom, P. P., F. Haesebrouck, R. Vandermeersch, J. Descamps, and K. Van Ommeslaeghe, 1991:
300 Prevalence of *Leptospira interrogans* serovar hardjo antibodies in milk in Belgian dairy
301 herds. *The Veterinary quarterly* 13, 118-120.
302 Ellis, W. A., and S. W. Michna, 1977: Bovine leptospirosis: experimental infection of pregnant
303 heifers with a strain belonging to the Hebdomadis serogroup. *Research in veterinary*
304 *science* 22, 229-236.
305 Ellis, W. A., J. J. O'Brien, S. D. Neill, and D. G. Bryson, 1986: Bovine leptospirosis:
306 experimental serovar hardjo infection. *Veterinary microbiology* 11, 293-299.
307 Evangelista, K. V., and J. Coburn, 2010: *Leptospira* as an emerging pathogen: a review of its
308 biology, pathogenesis and host immune responses. *Future microbiology* 5, 1413-1425.
309 Guerra, M. A., 2009: Leptospirosis. *Journal of the American Veterinary Medical Association*
310 234, 472-478, 430.
311 Hartman, E. G., P. Franken, B. A. Bokhout, and D. J. Peterse, 1989: [Leptospirosis in cattle;
312 milker's fever in cattle farmers]. *Tijdschrift voor diergeneeskunde* 114, 131-135.
313 Hartskeerl, R. A., and W. J. Terpstra, 1996: Leptospirosis in wild animals. *The Veterinary*
314 *quarterly* 18 Suppl 3, S149-150.
315 Herrmann, J. L., 1993: Genomic techniques for identification of *Leptospira* strains. *Pathologie-*
316 *biologie* 41, 943-950.
317 Jolley, K. A., and M. C. Maiden, 2010: BIGSdb: Scalable analysis of bacterial genome variation
318 at the population level. *BMC bioinformatics* 11, 595.
319 Levett, P. N., 2001: Leptospirosis. *Clinical microbiology reviews* 14, 296-326.

Levett, P. N., 2004: Leptospirosis: a forgotten zoonosis? *Clinical and Applied Immunology Reviews* 4, 435–448.

Levett, P. N., R. E. Morey, R. L. Galloway, D. E. Turner, A. G. Steigerwalt , and L. W. Mayer, 2005: Detection of pathogenic leptospires by real-time quantitative PCR. *Journal* of *Medical Microbiology* 54(1): 45-49.

Levett, P. N., 2015: Systematics of *Leptospiraceae* p. 11-20. In Ben Adler. *Leptospira* and Leptospirosis, Current Topics in Microbiology and Immunology. Springer Berlin Heidelberg.

Lin, M., O. Surujballi, K. Nielsen, S. Nadin-Davis, and G. Randall, 1997: Identification of a 35-kilodalton serovar-cross-reactive flagellar protein, FlaB, from *Leptospira interrogans* by N-terminal sequencing, gene cloning, and sequence analysis. *Infection and immunity* 65, 4355-4359.

Mayer-Scholl, A., E. Luge, A. Draeger, K. Nockler, and B. Kohn, 2013: Distribution of *Leptospira* serogroups in dogs from Berlin, Germany. *Vector borne and zoonotic diseases* 13, 200-202.

Morey, R. E., R. L. Galloway, S. L. Bragg, A. G. Steigerwalt, L. W. Mayer, and P. N. Levett, 2006: Species-specific identification of *Leptospiraceae* by 16S rRNA gene sequencing. *Journal of clinical microbiology* 44, 3510-3516.

Mori, M., M. Esbroeck, S. Depoorter, W. Decaluwe, S. J. Vandecasteele, D. Fretin, and M. Reynders, 2015: Outbreak of leptospirosis during a scout camp in the Luxembourg Belgian province, Belgium, summer 2012. *Epidemiology and infection* 143, 1761-1766.

OIE Terrestrial Manual, 2014: Leptospirosis. Available atw: http://www.oie.int/fileadmin/Home/fr/Health_standards/tahm/2.01.09_LEPTO.pdf (accessed 19 November 2015).

344 Ryan, E. G., N. Leonard, L. O'Grady, M. L. Doherty, and S. J. More, 2012: Herd-level risk
345 factors associated with *Leptospira* Hardjo seroprevalence in Beef/Suckler herds in the
346 Republic of Ireland. *Irish veterinary journal* 65, 6.
347 Smith, C. R., M. R. McGowan, C. S. McClintock, B. G. Corney, P. J. Ketterer, L. Smythe, and
348 W. Ward, 1997: Experimental *Leptospira borgpetersenii* serovar hardjo infection of
349 pregnant cattle. *Australian veterinary journal* 75, 822-826.
350 Terpstra, W. J., 1992: Typing *Leptospira* from the perspective of a reference laboratory. *Acta*
351 *Leidensia* 60, 79-87.

352 **Tables and figures caption**

353 **Table I.** List of pathogens included and diagnostic methods applied in the standardized panel
354 of analyses

Pathogens	**Fetus Samples**	**Methods**	**Foetal serum Methods**	**Maternal serum Methods**
Brucella spp.	Abomasal fluid	Culture		SAW /ELISA Ab
Campylobacter foetus spp.	Abomasal fluid	Culture		
Coxiella burnetii	Abomasal fluid	PCR**		ELISA Ab
Listeria monocytogenes	Abomasal fluid	Culture		
Mycotic agents	Abomasal fluid	Culture		
Opportunistic bacteria	Abomasal fluid	Culture $		
Salmonella spp.	Abomasal fluid	Culture		
BTV-8	Brain	PCR*		
Neospora caninum	Brain	PCR	ELISA Ab	ELISA Ab
Schmallenberg virus	Brain	PCR*		
BoHV-4	Spleen	PCR		ELISA Ab
BVDV	Spleen	ELISA Ag		ELISA Ab

	Leptospira serovar Hardjo	ELISA Ab

Legend: PCR, Polymerase chain reaction; Ab, Antibody; Ag, Antigen; SAW, Sero-agglutination of Wright; $, Only the presence of a pure culture on blood agar is indicative of opportunistic bacteria; *, Applied only if suspected case (congenital abnormalities); BVDV, Bovine Viral Diarrhoea Virus; BTV-8, Bluetongue virus serotype 8; BoHV-4, Bovine herpesvirus 4.

Table II. Distribution of MAT results among the tested cow sera according to different leptospiral serogroup (only serogroups with positive results are listed)

Titer	Grippotyphosa	Australis	Ictero-haemorrhagiae	Autumnalis	Panama	Pyrogenes	Sejroe	Cynopteri	Hebdomadis
Negative	25	29	36	35	37	40	39	39	41
1/160	4	2	4	2	2	1	2	3	
1/320	3	2	2	3	3	1			1
1/640	4	5		1					
1/1280	6	4		1			1		
Total positive	17	13	6	7	5	2	3	3	1
Total sera tested	42	42	42	42	42	42	42	42	42

366 **Table III.** Results of serological and antigenical analysis according to *Leptospira* serogroup

367 (only serogroups with positive results are listed, the highest titer of each serogroup is presented)

			Leptospiral serogroup								
ID	Pathogenic *Leptospira strains* (PCR)	Genotyping (MLST)	AUS	AUT	CYN	GRI	HEB	ICT	PAN	PYR	SEJ
CI-11-023436			-	-	-	-	-	-	-	-	-
CI-11-029715			-	-	-	-	-	-	-	-	-
CI-12-000889	**Pos**	***L. interrogans***	**640**	-	-	-	-	-	**640**	**320**	-
CI-12-001123			-	-	-	-	-	-	-	-	-
CI-13-006899			-	-	-	-	-	-	-	-	-
CI-13-011101			-	-	-	-	-	-	-	-	160
CI-13-018383			-	-	-	-	-	-	-	-	-
CI-13-019237			-	-	-	-	-	-	-	-	-
CI-13-022971			-	-	-	-	-	-	-	-	-
CI-13-032292			-	-	-	320	-	-	-	-	-
CI-13-032707			160	-	-	640	-	-	-	-	-
CI-14-034435	Pos		-	-	-	-	-	-	-	-	-
CI-14-034966	Pos		-	-	-	160	-	-	-	-	-
CI-14-037270	Neg		-	-	160	1280	-	-	-	-	-
CI-14-038297			1280	160	-	-	-	160	-	-	-
CI-14-038323			320	-	160	-	-	-	-	-	-
CI-14-038596	Pos		1280	1280	-	-	-	160	640	-	-
CI-14-040708	Neg		160	-	-	-	-	-	-	-	-
CI-14-042496			640	-	-	320	-	-	-	-	-
CI-14-042765			-	-	-	160	-	-	-	160	-
CI-14-044328	Neg		-	-	-	640	-	-	-	-	-
CI-14-044569	Pos		-	160	-	-	320	-	-	-	1280
CI-14-044707	Pos		-	-	-	640	-	-	-	-	-
CI-14-046867			-	-	160	1280	-	-	-	-	-
CI-14-047394	Pos		-	-	-	1280	-	-	-	-	-
CI-14-047531	Pos		1280	320	-	-	-	-	160	-	-
CI-14-047968	Pos		1280	320	-	-	-	320	-	-	-
CI-14-048785	Pos		640	-	-	-	-	-	160	-	-
CI-14-049712	Pos		640	640	-	-	-	320	640	-	160
CI-14-050512	Pos		-	-	-	-	-	-	-	-	-
CI-14-050870	Neg		-	-	-	-	-	-	-	-	-
CI-14-057066	Pos		-	-	-	-	-	-	-	-	-
CI-14-057564	Pos	*	-	-	-	640	-	-	-	-	-
CI-14-057925	Pos		-	-	-	-	-	-	-	-	-
CI-14-058607			-	-	-	1280	-	-	-	-	-
CI-14-058713	Pos		-	-	-	1280	-	-	-	-	-
CI-14-061536	**Pos**	***L. kirschneri***	**320**	-	-	**1280**	-	-	-	-	-
CI-14-062221	Pos	*	-	-	-	-	-	-	-	-	-
CI-14-063728	Pos	*	-	-	-	160	-	-	-	-	-
CI-14-065178	Pos		-	-	-	160	-	-	-	-	-

CI-14-067483	Pos		640	320	-	-	-	-	-	-	-
CI-14-069253	Neg		-	-	-	320	-	-	-	-	-

368

369 Legend:

370 * Unsuccessful amplification and sequencing; AUS, Australis ; AUT, Autumnalis ; CYN,

371 Cynopteri ; GRI, Grippotyphosa; HEB, Hebdomadis; ICT, Icterohaemorrhagiae ; PAN, Panama

372 ; PYR, Pyrogenes ; SEJ, Sejroe.

373

374 **Figure captions**

375 **Figure 1.** Geographical distribution of icteric abortion's case, years 2008-2014 (N=152)

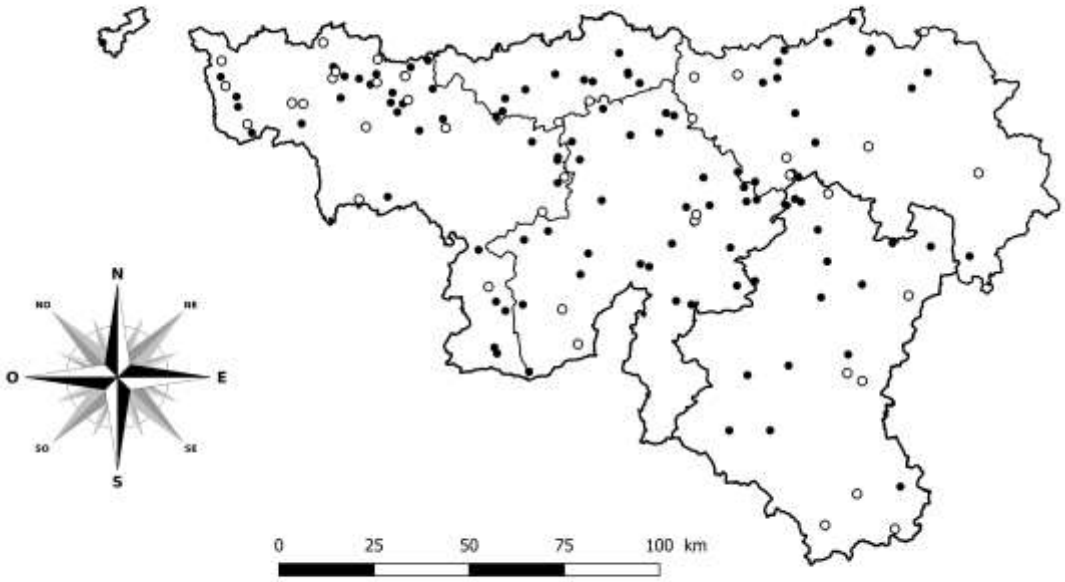
376 Legend: White dots correspond to icteric cases where complementary analysis (MAT and/or
377 RT-PCR) are performed; black dots corresponds to icteric cases without complementary
378 analysis.

379

380 **Figure 2.** Trends of icteric bovine aborted fetuses rate and the absolute number of notified
381 abortions

382

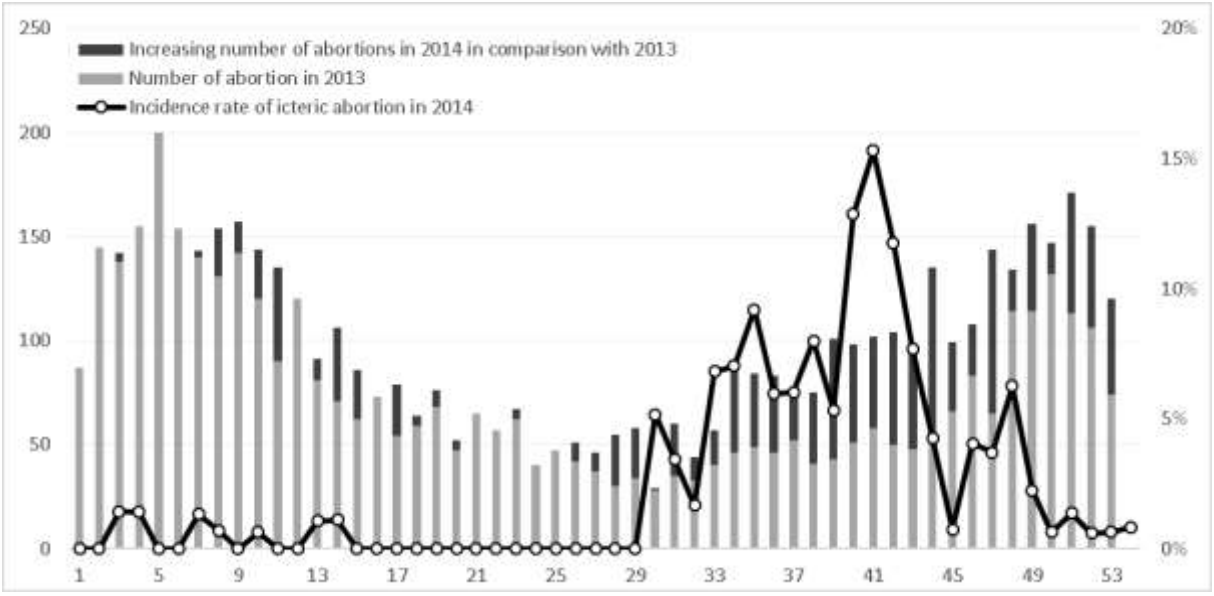
383 **Fig. 1**



384

385

386 **Fig. 2**



387

388

389