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Porcine brucellosis (*Brucella suis*)[1]

Scientific Opinion of the Panel on Animal Health and Welfare

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PANEL MEMBERS

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SUMMARY

Following a request from the European Commission (DG Health and Consumer Protection), the Panel on Animal Health and Welfare (AHAW) was requested for an opinion on porcine brucellosis (*Brucella suis*).

B. suis consists of five biovars, however infection in pigs is caused by the first three biovars (biovars 1, 2, and 3). Infection of animals caused by biovars 1 and 3 differs from that caused by biovar 2 in the host specificity and geographical distribution. In the context of public health, biovar 2 is very rarely pathogenic for humans, whereas biovars 1 and 3 are highly pathogenic causing severe disease in human beings.

There is currently no requirement for monitoring and surveillance of *B. suis* in domestic pigs or in wild life and therefore a lack of systematic epidemiologic data on porcine brucellosis in most MS. The occurrence of the disease is mainly sporadic (with the exception of certain areas where the characteristics of the production systems allow *B. suis* to be endemic). Within the EU, the epidemiological situation is varied, with some countries free of the disease, others reporting sporadic outbreaks and yet others reporting this disease as an emergent problem. Available epidemiological evidence shows that *B. suis* biovar 2 is the most common agent, but biovars 1 and 3 can also occur.

Available evidence also suggests that currently the wild boar seems to remain the main source of infection for domestic pigs because several outbreaks of *B. suis* occurred in outdoor rearing systems, even on fenced premises, with the source of infection traced to contacts with wild boars. Transmission from wild boars to pigs is thought to be through the venereal route, as

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crossed piglets (striped) have been reported, at least in France and Portugal. Other routes might also be possible. Hares have been considered as a possible source of *B. suis* outbreaks in domestic pigs via swill feeding with offal from hunted infected hares. Some reported outbreaks have also been traced to the introduction of infected live animals originating from holdings where the diseases had not been detected.

Based on the data of a systematic literature review, meta-analytical estimates of diagnostic sensitivity (Se) and specificity (Sp) of diagnostic tests for *B. suis* infection in pigs were generated. Highly sensitive and reasonably specific testing systems with the potential to combine more than one test are required for a rigorous detection and slaughter policy. Currently, serological testing in pigs is mainly useful to monitor the status of a herd but not reliable enough for single animals. Evidence from the systematic review suggests that indirect Enzyme-Linked ImmunoSorbent Assay (iELISA) and competitive Enzyme-Linked ImmunoSorbent Assay (cELISA) could be suitable candidates because of their high Se and Sp. However, the ELISA tests have not been fully evaluated and standardised for use in pigs. Primary reference standards are currently being developed. Formal procedures such as those implemented by the OIE should be considered for accreditation of candidate tests (*e.g.* iELISA and cELISA) for the purpose of control of *B. suis* in pigs. Little is known about the causes of false positive serological reactions to *B. suis* testing in pigs (FPSR), but it is believed that *Yersinia enterocolitica* O:9 could be the main factor of this problem. To address the FPSR issue it is important to improve the specificity of current diagnostic tests. Specific studies should also be conducted with the aim to identify the mechanisms of FPSR and to elaborate specific testing protocols to reduce this phenomenon. Further development of Brucellin-based tests should be encouraged since, in addition to bacteriology and molecular tools, these tests are the only confirmatory tests suitable to fully discriminate between true brucellosis infections and the infections caused by *Y. enterocolitica* O:9 or other cross-reacting bacteria.

The risk factors (RF) for *B. suis* introduction and spreading into domestic pigs (in particular through contact with wildlife, and subsequent spread within the EU by trade in pigs and pig semen) have been identified and qualitatively assessed. The presence of infected wild boars and hares and the potential for exposure of outdoor pig holdings remain the most important risk factors in the currently affected areas. Exposure to infected wild boar would be influenced by the level of biosecurity resulting in variable level of either direct or indirect contact. In addition to the level of biosecurity, direct contact would also be influenced by the type of pig housing (*e.g.* outdoor vs indoor). Should the infection become established in holdings participating to intra-Community trade (*e.g.* outdoor, indoor, semen collection centres), the most important risk factor for wider spread within the EU would the infection remains unrecognised. This would create the potential for further spread within the EU either by direct or indirect contact. Movement of live pigs (mainly breeding pigs) and semen would be the most important risk factor given the intensive level of intra-Community trade. Indirect contact would mainly depend on mechanical transmission by people and shared contaminated equipment. The role of other means of transmission (e.g. rodents, scavenging birds) remains hypothetical. Awareness should be raised in the pig industry for indicative clinical signs of porcine brucellosis and to the additional risk posed by illegal swill feeding including offal from hares and wild boars.

Semen production is well controlled by legal requirements related to the introduction of boars in semen collection centres, continuous monitoring of disease freedom and semen preparation requirements. However, transmission through this route could constitute an important way of

disease dissemination. Boars kept in semen collection centres should continue to be selected and introduced from holdings that are epidemiologically proven as free from *B. suis*. Donors should continue to be serologically tested on holding of origin and in quarantine before being placed in the centre as well as on a regularly basis afterwards. The results indicate that the iELISA and cELISA could have the potential of being used for testing of boars for admission to semen collection centres and for compulsory routine testing.

Key words: ***Brucella suis*, Brucellosis, Pig, Risk Assessment, Animal Health, Diagnostic tests, Meta-analysis, Intra-Community trade, Zoonosis.**

TABLE OF CONTENTS

TABLE OF FIGURES AND TABLES

BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION (DG HEALTH AND CONSUMER PROTECTION)

1.1. Epidemiology

Porcine brucellosis is a disease affecting domestic and feral pigs which constitute the main reservoirs. It is also a zoonosis, acquired from handling infected pigs. It is caused by a bacterium called *Brucella suis*. There are five different types of this bacterium, called biotypes, which behave in slightly different ways outside the pig.

In most parts of the world where *B. suis* infects pigs, the most common biotypes causing disease are l and 3, with the addition of biotype 2 in Europe. Biotype 2 is enzootic in wild boar and hare populations in Northern, Central Europe and South-Eastern Europe and these animal species transmit it to pigs. Porcine brucellosis has also been reported in Austria, France, Belgium, Germany, Croatia, Portugal and Spain.

B. suis is not present in the United Kingdom or Ireland. It is assumed that it is still enzootic in the hare populations of Scandinavia and Central Europe, but there is insufficient evidence to define the precise area where infected hares live. It is also present in the USA, South America, parts of Asia and Australia.

Once porcine brucellosis is introduced into a pig herd, it is difficult to eliminate. It causes long-term reproductive losses and some biotypes (1 and 3 particularly) also cause a very serious disease in humans. Fortunately, the hare biotype-type 2 is less pathogenic to humans when transmitted.

1.2. EU Legislation

1.2.1. Food Law ("Hygiene Package")

In EU Food Law, brucellosis in animals is listed as a specific hazard and detailed provisions for the disease to ensure safety of meat and to protect public health have been established therein. Chapter IX(F) of Section IV of Annex I to Regulation (EC) No 854/2004 of the European Parliament and of the Council of 29 April 2004 lays down specific rules for the organisation of official controls on products of animal origin intended for human consumption[2] *i.e.*:

1. When animals have reacted positively or inconclusively to a brucellosis test, or there are other grounds for suspecting infection, they are to be slaughtered separately from other animals, taking precautions to avoid the risk of contamination of other carcases, the slaughter line and staff present in the slaughterhouse.
2. Meat from animals in which post-mortem inspection has revealed lesions indicating acute infection with brucellosis is to be declared unfit for human consumption. In the case of animals reacting positively or inconclusively to a brucellosis test, the udder, genital treat and blood must be declared unfit for human consumption, even if no such lesion is found.

1.2.2. Imports to the Community of live pigs and pig meat

Moreover, Council Decision 79/542/EEC of 21 December 1979 drawing up a list of third countries or parts of third countries, and laying down animal and public health and veterinary certification conditions for importation into the Community of certain live animals and their

[2] OJ L 155, 30.4.2004, p206

fresh meat[3] as regards imports of pigs for breeding and production[4] and fresh pig[5] meat, sets up specific regimes to be applied with respect to porcine brucellosis.

1.2.3. Intra-Community trade in pigs

As regards intra-Community trade in porcine animals, Council Directive 64/432/EEC of 26 June 1964 on animal health problems affecting intra-Community trade in bovine animals and swine[6] introduced the obligation to certify pigs as originating from brucellosis-free herds and substantiating a test regime to be applied in order to obtain such a status.

However, due to the technical development in pig husbandry, those requirements were removed from that Directive by Directive 97/12/EEC of 17 March 1997 amends and updates Directive 64/432/EEC on health problems affecting intra-Community trade in bovine animals and swine[7].

The disease was thought to have disappeared from some Member States as no clinical cases had been diagnosed for a number of years. Then, over recent years, outdoor breeding pig herds were established which were exposed to wild hares. As a result pigs have caught brucellosis from infected hares.

1.2.4. Reporting and results

Currently, *Brucella suis* infection is listed in Annex E(II) of Directive 64/432/EEC as a notifiable disease and Member States are obliged to report annually on its occurrence within their territory in accordance with Article 8 of the Directive. In the last few years the tendency to reporting more cases has been observed.

Reporting period	*Number of cases*	*Reporting Member States*
2004*	58	AT, DE, HU, IT
2005**	72	FR, HU, IT
2006***	2	FR
2007**	39	IT

* 55 isolates obtained from wild boars within a surveillance programme in place in Italy (regions of Piemonte and Liguria)
** 63 isolates obtained from wild boars within a surveillance programme in place in Italy (regions of Piemonte and Liguria)
*** no data provided by Italy.
**** 22 isolates obtained from wild boars within a surveillance programme In place in Italy (regions of Piemonte and Liguria)
There are no cases and positive tests for BS infection in BG in 2007

Taking into account this trend and due to the recent enlargement of the European Union with new Member States where the free range system of keeping pigs is common, the risk of contact of domestic pigs with wild boars and hares is very high.

Porcine brucellosis is a rarely reported disease in the EU. Seventeen Member States reported testing of 37,819,547 pigs, of which 21 pigs were positive for *Brucella* spp.[8] In Hungary, *Brucella* was not detected in 5,730 tested pig herds.

In 2006, *Brucella suis* was isolated from domestic pigs by bacteriological tests in Belgium and Germany. In addition, *Brucella suis* was also detected in hares in the Czech Republic, Hungary and Spain and isolated from wild boars in Italy.

[3] OJ L 146, 14.6.1979, p. 15. Decision as last amended by Decision 2008/6l/EC (OJ E 15, 18.1.2008, p. 33).
[4] Annex I, Part 2, Point 10.4.C and 10.5 of the health certificate POR-X
[5] Annex 11, Part 2, paint 10.3(b) and (c) of the health certificate POR
[6] OJ121,29.7.1964, p.1977/64
[7] OJ L 109, 25.4.1993, p. 1-37
[8] http://www.efsa.europa.eu/EFSA/Documentset/Zoon_rep_2006_en,0.pdf

1.2.5. Porcine semen

Moreover, Council Directive 90/429/EEC of 26 June 1990 laying down the animal health requirements applicable to intra-Community trade in and imports of semen of domestic animals of the porcine species[9] establishes compulsory testing schemes for donor boars with respect to porcine brucellosis in the semen collection centres. Testing methods should be assessed taking into account new technical developments.

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION (DG HEALTH AND CONSUMER PROTECTION)

In view of the above, and in accordance with Article 29 of Regulation (EC) 178/2002 and Article 20(2) of Regulation (EC) No 854/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption, the Commission asks EFSA to provide scientific advice on:

- the significance of the presence, origin and occurrence of brucellosis in pigs (*Brucella suis*) in the EU for a better understanding of the impact of the disease in the context of the new epidemiological situation;
- the risk of porcine brucellosis (*Brucella suis*) being introduced into domestic pig herds, in particular through movement of and trade in pigs and contact with wildlife; and assessment of the risk factors for such introduction and spread of the disease;
- the appropriateness of the current measures, different elements and possible strategies that can be used to control and fight against brucellosis in pigs (*Brucella suis*);
- the suitability of available tests for porcine brucellosis (*Brucella suis*).

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[9] OJ L 224, 18 .X. 1990, p. 62-72

ASSESSMENT

1. Introduction - Approach for this Mandate

The mandate for this scientific assessment focuses on *Brucella* (*B.*) *suis* as hazard is addressed in the following steps. A brief description of the hazard is given with emphasis on aspects relevant for a qualitative risk assessment of the current situation of *B. suis* in the European Union (EU) Member States (MS) (Chapter 2). This is to address the 1st ToR on the relevance of *B. suis* in the EU. The epidemiology of *B. suis* is described in terms of geographical occurrence, the role of wildlife and routes of transmission under acknowledgment of uncertainties arising from incomplete scientific information (Chapter 3). The pathogenesis (Chapter 4), clinical signs (Chapter 5) and diagnostic tools (Chapter 6) are a summary, again with emphasis on aspects relevant for the risk assessment. A systematic review of available scientific data on the diagnostic performance of tests for *B. suis* in pigs along with a statistical meta-analysis of the diagnostic sensitivity and specificity has been conducted by the working group and it is reported in the document (Chapter 7). Chapters 6 and 7 address the 4th ToR on the suitability of tests. Risk pathways for the hazard of concern have been elaborated using expert knowledge available in the working group. In relation to these pathways, risk factors have been identified and assessed qualitatively (Chapter 8). Despite the qualitative approach, efforts were made to capture variability (*e.g.* due to different epidemiological situations encountered in MS) and uncertainty (*e.g.* as evident from scores elicited independently from the experts) of this assessment. The results of the qualitative risk assessment address the 2nd ToR on risk factors for introduction and spread of the hazard. Finally, conclusions will be drawn from material presented in various Chapters to assess the potential value of control options (Chapter 9). These science-based conclusions will address the 3rd ToR on the appropriateness of current measures, different elements and possible strategies. For the purpose of this Opinion, a case definition of Brucellosis for domestic or wild pig (*Sus scrofa*) populations has been adopted by the WG, which is further elaborated in Chapter 8.

2. Description of the causative agent (*B. suis*)

Hutyra as early as 1909 isolated a species of *Brucella* from foetuses of aborting sows in Hungary (Huddleson, 1929). The agent was also isolated from aborted porcine foetuses in the USA in 1914 (Traum, 1914). For many years it has been thought to be caused by an exceptionally pathogenic form of *Brucella abortus* (Alton, 1990). In 1929, *Brucella suis* was nominated as a separate species (Huddleson, 1929). To date (June 2009), there are five recognised biovars of *B. suis* (1-5) (OIE, 2008a).

2.1. Morphology (and biovars)

Brucella organisms are Gram negative, coccobacilli, usually arranged singly, but they may be in pairs or small groups. The length varies from 0.6 μm to 1.5 μm and the width from 0.5 μm to 0.7 μm. The morphology is fairly constant and pleomorphic forms are rare except in old cultures. The disease caused by biovars 1 and 3 is similar, while that caused by biovar 2 differs from the others in its host range and pathogenicity. Biovar 2 is very rarely pathogenic for humans, whereas biovars 1 and 3 are highly pathogenic causing severe disease (Alton, 1990; OIE, 2008a). These three biovars usually occur in nature in the smooth form.

2.2. Uniqueness of B. suis in relation with other Brucella species

There are nine recognized species of *Brucella* (Euzeby, 2009; National Centre for Biotechnology Information, U.S. National Library of Medicine 2009) that differ in their host preference:

- *B. abortus* preferentially infects cattle;
- *B. melitensis* preferentially infects sheep and goats;
- *B. suis* preferentially infects pigs;
- *B. canis* infects the dog;
- *B. ovis* infects sheep;
- *B. neotomae* has been only reported in the desert wood rat;
- *B. microti* has been firstly identified in the common vole (Scholz *et al*, 2008a; 2008b);
- *B. ceti* and *B. pinnipedialis*, have been mainly isolated from cetaceans and seals respectively (Foster *et al.*, 2007).

Some of the above mentioned species are subdivided into biovars according to classical laboratory techniques. The correct identification of the different species and biovars is essential for accurate interpretation of the epidemiological information during the outbreaks of the disease.

While pigs (*Sus scrofa*) are primarily infected by biovars 1, 2 and 3 of *B. suis*, porcine brucellosis may also be due to *B. abortus* or *B. melitensis* in areas where brucellosis is enzootic in ruminants.

B. suis biovar 4 infects reindeer, caribou, moose, bison, arctic foxes and wolves. *B. suis* biovar 5 has been reported in wild rodents in the former USSR (OIE, 2008a).

Moreover, *B. suis* can infect cattle (Cook and Noble, 1984; Forbesand and Tessaro, 2003; Garin-Bastuji and Delcueillerie, 2001), dogs (Barr *et al*., 1986), horses (Cvetnic *et al*., 2005) and humans (Hall, 1990). The biovar 2 is very rarely reported in cattle (Garin-Bastuji and Delcueillerie, 2001) and small ruminants (Garin-Bastuji, personal communication, March 2009), and in humans (Teyssou *et al.*, 1989; Paton *et al.*, 2001; Garin-Bastuji *et al.*, 2006).

Some other animals are also susceptible to *B. suis*: Muskox (*Ovibos moschatus*) is susceptible to *B. suis*, biovar 4 (Forbes, 1991). This is a wild Arctic mammal of the *Bovidae* family. Muskoxen are native to the Arctic areas of Canada, Greenland, and Alaska. The species has been introduced also in Sweden, Estonia, Norway and Russia. Pecaries (Javelinas) are *Suidae* like animals from family *Tayassuidae*. *B. suis* biovar 1 has been isolated from these animals in Venezuela (Lord and Lord, 1991). *Ovibos moschatus* (Muskox) and *Tayassuidae* family (Pecaries) are included as susceptible to *B. suis* animals in Annex A of Directive 92/65/EEC (EC, 1992).

2.3. Antigenic characteristics

All smooth forms of *Brucella* species react in agglutination tests with antisera prepared against smooth *Brucella* cultures. Morphologically related Gram-negative organism that could be confused with *Brucella*, are not agglutinated by these antisera. However, some Gram-

negative bacteria (*Yersinia enterocolitica* O:9 is probably the most frequent cause) can also cross-react with antisera raised against smooth *Brucella* spp.

Similarly to other gram-negative bacteria, the outer membrane of *B. suis* is composed of phospholipids, proteins and smooth lipopolysaccharide (S-LPS). The S-LPS is the immunodominant antigen and antibodies induced in the host by *B. suis* infection are mainly and the most frequently directed against this S-LPS. Therefore, most serological tests, particularly those using whole-cell suspensions as antigen such as the Rose bengal test (RBT) and the Complement Fixation test (CFT), and most immunosorbent assays, have been developed to detect antibodies to this antigen. The S-LPS is composed of an inner glycolipid moiety (containing the core oligosaccharide plus the lipid A) and of an outer polysaccharide chain (O-chain). This O-chain is the relevant antigenic moiety in *B. suis* and it is chemically composed by a perosamine homopolymer showing mainly α-1,2 linkages.

In addition to the S-LPS, several outer membrane proteins (OMP) are also exposed in the surface of *B. suis*. These antigens can be extracted from the *B. suis* outer membrane and used as diagnostic antigens. However, the resulting tests are less sensitive than those using S-LPS as antigen. The cytoplasmic proteins are internal antigens, not exposed to the outer bacterial surface. These inner proteins are considered specific of the genus *Brucella* and show little antigenic differences between the several *Brucella* species. These inner antigens, known also as brucellin or brucellar allergen, can be used for allergic diagnosis of brucellosis in swine, being very useful to differentiate infections due to *Brucella* spp. from those due to bacteria whose LPS cross-reacts with the *Brucella* S-LPS, as is the case of *Yersinia enterocolitica* O:9.

2.4. Molecular characteristics

Two complete *B. suis* genomes were sequenced and annotated, *B. suis* biovar 2 (ATCC 23445) and *B. suis* biovar 1 strain 1330 (ATCC 23444). Two other *B. suis* genomes have been sequenced and are being annotated, they are *B. suis* biovar 3 strain 686 (ATCC 23446) and *B. suis* biovar 5 strain 513 (National Centre for Biotechnology Information, U.S. National Library of Medicine, 2009).

The *B. suis* genome strain 1330 was studied by Paulsen *et al.* (2002), the genome was found to consist of two circular chromosomes of 2,107,792 bp (Chr I) and 1,207,381 bp (Chr II). Like the other *Brucella* genomes studied, it has a G-C content of 57-58% (National Centre for Biotechnology Information U.S. National Library of Medicine, 2009), a total of 2,185 and 1,203 Open Reading Frames (ORFs) were identified on Chr I and II (Paulsen *et al.*, 2002). Upon comparison of the *B. suis* genome to the genome of *B. melitensis* the majority of the genes (>90%) share 98–100% identity at the nucleotide level, variable genes consisted primarily of hypothetical genes (Paulsen *et al.*, 2002). Upon comparison of the *B. suis* genomes to other genomes of the alpha-proteobacteria such as *Bartonella* spp., *Agrobacterium* spp., *Ensifer* spp. and others, it was found that a total of 1,902 ORFs of *B. suis* were conserved in three genomes; *Mesorhizobium loti*, *Sinorhizobium meliloti*, and *A. tumefaciens*, and that 2,408 *B. suis* ORFs were conserved in at least one of these three genomes (Paulsen *et al.*, 2002). It was found that *B. suis* has transport and metabolic capabilities similar to those of soil and plant associated bacteria. It was hypothesized that these functions probably contribute to the survival of *B. suis* outside of its host and that there could probably be similarities in parasitic/symbiotic strategies between animal pathogens such as *B. suis* and plant pathogens such as *A. tumefaciens* or plant symbionts such as *S. meliloti* (Paulsen *et al.*, 2002).

Studies with whole genome analysis conducted by Chain *et al.* (2005) have suggested that because of distribution of pseudogenes, deletions, and insertions due to possible genomic rearrangements specie-specific DNA sequences, and distinct patterns of gene inactivation, *B. abortus* and *B. melitensis* share a common ancestor that diverged from *B. suis* which has undergone fewer genetic mutations (Chain *et al.*, 2005). The authors also commented that the above observation probably explains why *B. melitensis* and *B. abortus* seem to be more restricted in host range with the potential to cause abortion in sheep, goats, cattle, and all members of the clade Ruminantiae whereas, unlike other *Brucella* spp., *B. suis* appears to be the most diverse in genomic structure and host preference and can infect a broader range of animals (swine, reindeer, rabbits, and dogs) (Chain *et al.*, 2005, Moreno and Moriyón, 2001).

The definition of genome sequences of the different *Brucella* is of crucial importance since it would enhance the knowledge on the biochemical pathways of the bacterium and would allow the identification of virulence. Moreover, this knowledge would help to clarify the *Brucella* host-specificity, and to develop new diagnostic tests for the eradication of the disease (Bannantine and Paustian, 2006). The taxonomic position of *B. suis* within the genus *Brucella* is the subject of an ongoing debate, complicated by the high level of relatedness displayed by members of the *Brucella* genus in general. A number of genetic observations supported by independent studies have demonstrated that, with the exception of *B. suis* biovar 5, all *B. suis* and *B. canis* strains form a consistent group of organisms within the *Brucella* cluster (Fretin *et al.*, 2008). Paulsen *et al.* (2002) defined a series of differences responsible for the diversities in virulence and host preference between *B. suis* and *B. melitensis* by comparing their strictly related genomes (Paulsen *et al.*, 2002).

Conclusions

Brucella suis consists of five biovars, however infection in pigs is caused by the first three biovars (biovars 1, 2, and 3). Infection of animals caused by biovars 1 and 3 differs from that caused by biovar 2 in the host specificity and geographical distribution. In the context of public health, biovar 2 is very rarely pathogenic for humans, whereas biovars 1 and 3 are highly pathogenic causing severe disease in human beings. According to morphological characteristics and colony morphology on solid media, *B. suis* strains are indistinguishable from the other smooth *Brucella* species.

Like the other smooth *Brucella* species, *B. suis* reacts in agglutination tests with antisera raised against smooth *Brucella* cultures. The outer membrane of *B. suis* is mainly composed of phospholipids, proteins and smooth lipopolysaccharide (S-LPS). The S-LPS is the immunodominant antigen and most serological tests have been developed to detect antibodies to this antigen. Several outer membrane proteins (OMP) can be used as diagnostic antigens but the resulting tests are less sensitive than those using S-LPS. The cytoplasmic proteins are internal antigens considered specific for the genus. These inner antigens, known also as brucellin or brucellar allergen, can be used for allergic diagnosis of swine brucellosis, being useful to differentiate infections due to *Brucella* from those due to *Yersinia enterocolitica* O:9.

3. Epidemiology of porcine brucellosis (*B. suis*)

3.1. Geographical distribution

According to the World Organization for Animal Health (OIE) - World Animal Health Information database (WAHID) (OIE, 2009) (the global animal health reporting system), *B. suis* is reported in most continents. Generally, sporadic cases are reported in domestic pigs, but in some regions, such as South America and South-East Asia, the reporting rates are higher. Porcine brucellosis may be present, but currently unrecognised in some countries. Overall, available scientific literature has not shown widespread patterns related to geographic areas where outbreaks of brucellosis occur.

3.1.1. Global distribution

In the USA, *B. suis* has been successfully eradicated from the domestic pig population. However, the feral pig population still harbours the infection (Edmonds *et al.*, 2001).

B. suis has been isolated from pigs and humans in all central American countries (Moreno, 2002). Isolation of *B. suis* biovar 1 has been reported in Mexico (Luna-Martínez and Mejía-Terán, 2002).

B. suis has been identified as a cause of abortions in pigs also in central Venezuela (Vargas, 2002). In Brazil, *B. suis* is the second most prevalent *Brucella* infection. Only *B. suis* biovar 1 was reported as isolated in this country and there have been few surveys specific to pigs, although antibody prevalence seemed to decrease from 1981 to 2000 because of intensification and integration of pig production in large industrial clusters (Poester *et al.*, 2002). RBT is used as the screening test, confirmed with CFT or 2-mercaptoethanol (MAPA-Brasil, 2002). In Argentina, *B. suis* biovar 1 is frequently isolated from pigs. RBT and Buffered Plate Agglutination Test (BPAT) are used as screening tests and 2-mercaptoethanol as a confirmatory test (Samartino, 2002). Isolation of *B. suis* biovar 1 has been reported also in Paraguay from a pig herd were abortion occurred (Baumgarten *et al.*, 2002).

Isolation of *B. suis* has been reported in pigs and humans in 21 provinces of China (Deqiu *et al*. 2002).

In Japan, testing of serum samples from 115 wild boars for antibodies to *B. suis* using the Tube Agglutination Test (TAT) and the Enzyme-Linked Immunosorbent Assay (ELISA) resulted in 7.8% positive results (Watarai *et al.*, 2006). However, no case has ever been reported in domestic pigs (OIE, 2009).

B. suis is considered endemic in feral pigs in central Queensland, Australia. Infection in domestic pigs and cattle have also been recorded (Mason and Fleming, 1999).

In Africa, the disease occurs sporadically. In Egypt *B. suis* is present (biovar 1 was reported), although often unrecognized and unreported (Refai, 2002). Sub-Sahara African countries that officially reported porcine brucellosis between 1996 and 2000 include Côte d'Ivoire in West Africa, Central African Republic in central Africa, Uganda in east Africa and Mozambique in southern Africa. In addition, Mali, Nigeria and Democratic Republic of Congo (then Zaire) have all previously reported the disease (McDermott and Arimi, 2002). However, no mention of the biovar involved was found in the available literature.

3.1.2. Distribution of *B. suis* occurrence in Europe

3.1.2.1. *B. suis* in domestic pigs

Historical data indicate that brucellosis in swine had a sporadic or endemic occurrence in several European countries in the 1950ies (Thomsen, 1959). According to current available data, *B. suis* in domestic pigs has never been reported in Finland, Sweden, Norway and the United Kingdom (Godfroid and Käsbohrer, 2002). Sporadic cases of *B. suis* infection in domestic pigs have been reported in Germany, France, Denmark, Austria, Portugal and Spain (Godfroid and Käsbohrer, 2002; Appendix 1). Demonstrated clinical disease has been also reported recently in Romania, Czech Republic, Croatia, Serbia and Montenegro (OIE, 2009).

Although infections due to *B. suis* biovar 1 and 3 have been reported in several animal species and humans in Europe (Godfroid, 2002; Cvetnic *et al*., 2005), the most common *B. suis* biovar isolated in Europe is biovar 2. Historically, the geographical distribution of *B. suis* biovar 2 has been considered in a broad range between Scandinavia and the Balkans. This biovar is considered of low pathogenicity for humans, which are more frequently infected by the biovars 1 and 3. However, several human cases due to *B. suis* biovar 2 have been reported (Teyssou *et al*., 1989; Paton *et al*., 2001; Lagier *et al*., 2005; Garin-Bastuji, 2006).

Data on *Brucella* in pigs in the EU are collected by the EFSA and reported in the Annual Zoonosis Report (EFSA, 2006a; EFSA, 2007; EFSA, 2009). It should be emphasised that there is no legal obligation for MS to test the standing population of pigs for *Brucella* infection. Therefore, there is no common and harmonised basis for monitoring *B. suis* in the EU. Moreover, considerable methodological variation exists regarding the detection and confirmation of *B. suis*. When assessing data of the Annual Zoonosis Report, it should be considered that not all reported positive cases are consistent with the case definition relevant for international trade purposes, *i.e.* with either bacteriological or epidemiological confirmation of serological reactors.

3.1.2.2. *B. suis* in wildlife species

Isolation of this biovar has been primarily reported in two wild animal species: wild boars and hares. An overview of the ecology and distribution of these wildlife species is reported in Appendix 2.

Wild boars

In recent sporadic and limited outbreaks in Europe, wild boars have been identified as the potential source of transmission of biovar 2 to outdoor or extensively reared pigs. The presence of this infection in wild boars has been reported in many parts of Europe (Dimitrov *et al.*, 1977; Mineva *et al.*, 1991a; Godfroid *et al.*, 1994; Garin Bastuji *et al*., 2000; Hubálek *et al*., 2002; Taleski *et al*., 2002; Cvetnic *et al*., 2003; Cvetnic *et al*., 2004; Vaz *et al.*, 2004; Ruiz-Fons *et al*., 2006; Melzer *et al.*, 2006; Leuenberger *et al*., 2007; Szulowski *et al.*, 2008).

Hares

In Europe brucellosis in hares was first reported from Germany (Witte, 1941) and later from Switzerland (Roux and Bouvier, 1946) France (Jacotot and Valée, 1951) and former Czechoslovakia (Bouvier et al., 1954). In Denmark the first case was identified in 1951 and due to epidemiologic links with outbreaks of brucellosis in swine studies on the occurrence in hares were performed. In 1954 significant serological reactions were found in 35 out of 613

shot hares out of which *B. suis* was isolated from 16 which also had lesions typical for brucellosis (Bendtsen et al., 1956). During 1954-55 blood samples were investigated serologically from 1941 shot hares out of which 82 (4.2%) showed positive reaction (Christansen and Thomsen, 1956). The *Brucella* strains isolated from hares were also experimentally transmitted to and found to be highly pathogenic to swine following peroral infection. During 1929-99 Denmark also experienced 10 clinical outbreaks or serological reaction against *B. suis* in swine which during later outbreaks were verified as biovar 2. Due to epidemiological evidences the sources of these outbreaks have been linked to contact with hares. Swill feeding with offal from hunted infected hares, were considered as the major and the most likely route of transmission (Bendtsen et al., 1956). Education of hunters and intensive efforts to prevent swill feeding is considered a major reason to the decrease in outbreaks in swine originating from hares. However, it is likely that the prevalence of the infection in hares can be a link also to the infection in wild boars. Data from surveys of the hare population in different MS have not been found but for example in France brucellosis is considered to be endemic in hare populations (Appendix 1). The presence of this infection in hares has also been reported in different parts of Europe (Quaranta et al., 1995; Szulowski, 1999; Treml et al., 2007; Szulowski et al., 2008).

3.2. Survival of *B. suis* in the environment

There is no information on the specific survival characteristics of *B. suis* compared to other *Brucella* species. *B. abortus* and *B. melitensis* are generally considered as, among the non-sporulating Gram-negative bacteria, the most resistant outside their natural host.

Survival of *Brucella spp*. in the environment is increased with cold temperatures and moisture. *Brucella* survives up to 4 months in damp soil, water, urine and milk (Hirsh and Zee, 1999). In carcasses and organs *Brucella spp*. can survive up to 135 days and in blood at 4°C, 180 days (PHA Canada, 2009). Animal premises and pastures may remain contaminated for period up to two years but direct sunlight reduces the survival (HPA UK, 2009). *Brucella* can withstand drying and also survive in aborted foetuses, manure, wool, hay, dust, equipment and clothes (CFSPH-YSU, 2007). *Brucella* is destroyed by pasteurisation or cooking.

As *B. suis* is concerned, there is no report showing a specific difference among biovars. However, the biovar 2 appears as particularly sensitive outside the host compared to *B. abortus* and *B. melitensis*. In the laboratory it is common to isolate very few colonies of *B. suis* biovar 2 from infected pigs, wild boars or hares samples. Moreover, this biovar does not survive as long as *B. abortus* and *B. melitensis* in tissue samples stored frozen. Therefore it could be assumed that at least the biovar 2 of *B. suis* does not survive outside its host as long as classically described for other *Brucella* (Garin-Bastuji, personal communication, March 2009).

3.3. Transmission of *B. suis*

3.3.1. Host susceptibility

Pig brucellosis seems to affect both sexes equally and age does not have a major influence in susceptibility (Alton, 1990). Cameron *et al*. (1942) found a difference in hereditary resistance between pig families (Duroc-Jersey crosses) challenged with *Brucella*, postulating the existence of recessive genes for resistance to infection.

3.3.2. Routes of transmission

In domestic pigs, *B. suis* infection can spread from one infected animal within few months to 50% of the animals of a herd and infection rates of up to 80% are not uncommon (Beer, 1980; Szulowsky, 1999; Garin-Bastuji, personal communication, March 2009). Without being serologically diagnosed, the disease in endemic areas with mild clinical signs, can be unnoticed in the herd for a long time.

The infection routes are mainly oral (e.g., ingestion of aborted foetuses, foetal membranes and contaminated foodstuffs) (OIE, 2008a), but also venereal (Metcalf et al., 1994) (e.g., infected boars are often not infertile and could significantly contribute to the spread of the disease; artificial insemination (AI) with contaminated semen is another possibility) or conjuctival-mucosal (Acha and Szyfres, 1991). The minimum infectious dose for oral infection appears not to be known. Infection can also be transmitted from infected sows to their piglets either transplacentary (i.e., being born infected) or ingesting the bacteria in their mother's milk (Alton, 1990) or via contaminated environment. However, infection is usually temporary in suckling pigs and few retain infection and become carriers (Acha and Szyfres, 1991).

3.3.3. Infectious dose

No precise information has been published on the doses required to infect 100% of challenged pigs from different breeds and reared under different husbandry systems. However, doses as low as 10^4-10^5 colony-forming unit (CFU) appear to be sufficient to infect most of pigs challenged by the conjunctival route, but the severity of the infection was not correlated with dose, nor with the route of inoculation (Cedro *et al.*, 1971).

3.3.4. Transmission from holding to holding

Risk factors associated with transmission of *B. suis* between holdings or with the introduction of the infection in a pig production unit are revised and detailed in Chapter 8. The main factors associated with the introduction of porcine brucellosis in pig herds are the introduction of an infected live animal, contact with wildlife reservoirs, use of contaminated semen or feed (Alton, 1990) or the use of a communal boar. Other possible factors are the introduction of contaminated transport means, holding equipment and utensils and the introduction of infected offal (*e.g.* placenta and afterbirths).

Limited knowledge on transmission routes involving vectors such as dogs, cats, migrating wild birds, feed, water or litter (straw) is available (Körmendy and Nagy, 1982; Pikula *et al.*, 2005; Pawlow *et al.*, 1960). Fodder and straw contaminated by infected wildlife (hares and wild boars) may be a source of transmission (Dedek, 1997).

The high rate of infection of wild boars in Europe, represents a risk for spreading the infection to domestic pigs and, to a lesser extent, a source of infection for other mammalian species, including humans. This has been the source identified in outbreak investigation in several MS, where biosecurity of production systems are low either by free ranging of pigs (as in Portugal and Spain) or by an "open-air" system of commercial holding. The role of wild boar hunting (migration pressure, remains of faeces and lochia remaining in the field, hunters working on the premises, etc.) has not been fully investigated.

3.3.4.1. Semen

Boars infected with *B. suis* biovar 1 may shed 10^4-10^5 CFU per ml semen (Lord *et al.*, 1998), and thus spread the infection.

The conditions for approval and supervision of semen collection centres are outlined respectively in Chapters I and II of Annex A to Council Directive 90/429/EEC of 26 June 1990, laying down the animal health requirements applicable to intra-Community trade and imports of semen of domestic animals of the porcine species. The conditions applying to the admission of animals to approved semen collection centres set up in Chapter I of Annex B to Directive 90/429/EEC, include the sourcing from herds "*free of brucellosis in accordance with the Article 3.5.2.1.* (now 15.4.2.) *of the OIE International Animal Health Code* (now Terrestrial Animal Health Code)" and testing of the animals for brucellosis on samples collected during pre-entry quarantine.

Compulsory routine testing for animals kept at an approved semen collection centre are explained in Chapter II of Annex B to the Directive, and they include testing for Aujeszky's disease, Classical Swine Fever and Brucellosis, on 25% of the animals every three months. All animals should be tested at least once during their stay at the centre and at least every 12 months if their stay exceeds a year. In Chapter 9, the current and alternative testing protocols in relation to semen collection centres will be further explored.

The Buffered *Brucella* Antigen Test is currently the only authorised test.

Following the completion of the single market, Directive 64/432/EEC on animal health problems affecting intra-Community trade in bovine animals and swine (EC, 1964) was amended by Directive 97/12/EC of 17 March 1997. In anticipation of the new provisions on porcine brucellosis in Directive 97/12/EC which would, following the adoption of Directive 98/46/EC, become applicable as of 1 July 1999, Directive 98/99/EC of 14 December 1998 discontinued compulsory brucellosis testing of pigs for breeding and production intended for intra-Community trade as of 1st January 1999. However, during the first six months until 1 July 1999 this Directive continued requiring that swine for breeding or production must be brucellosis-free and come from brucellosis-free stock.

Semen is collected following hygienic, traceability and quality control procedures. Semen collection uses a dummy (no females involved) and disposable materials to avoid contamination when it is used as fresh semen (preserved at 16-18 ^{0}C), within 1-5 days from collection. Semen diluents are added shortly after collection and containing nutrients (extenders), stabilizers and may contain antibiotics.

According to Council Directive 90/429/EEC, the antibiotic combination added to the semen must produce an effect at least equivalent to the following final dilutions of semen: 500 µg Streptomycin/ml; 500 IU Penicillin/ml; 150 µg Lincomycin/ml; 300 µg Spectinomycin/ml. Of this combination, only Streptomycin could have potential inhibitory effects on *B. suis*.

Many antibiotic combinations are used in commercial mixtures, Penicillin-Streptomycin and Lincomycin-Spectinomycin being the preferred ones.

Commercial antibiotic mixtures can also combine other antibiotics. Some examples are: Penicillin and Neomycine, in some cases added with Gentamycin (Schippers); Colistin (33 mg/l) and Neomycine (83 mg/l) with Enrofloxacin, Cephalosporins or Gentamycin added according to customer's requirements (Kubus); Neomycin sulphate (1 g/l) (Boar Semen Extender BTS, Minitube), Gentamicin sulphate (250 mg/l) (Boar Semen Extender-Merck III).

From these mixtures Gentamycin is the antibiotic with most inhibitory effect for *B. suis* (Rolain *et al.*, 2000). Brucellosis infection can not be eliminated by the use of antibiotics in semen, because the amount of antibiotic required need to be high what may be incompatible with semen survival. Moreover, in some particular conditions, survival of *B. suis* can also be possible (*i.e.*, the presence of inflammatory cells in semen with intracellular *Brucellae*).

Conclusions and recommendations

Conclusions and recommendations relevant for this section are listed in Chapter 9.

4. Pathogenesis of *B. suis* infection

4.1. Phases of infection

The pathogenesis of *B. suis* in pigs has not yet been explained fully. The sequence of events following the entry of *B. suis* is supposed to be similar to that described during other brucellosis infections in different animal species. There is generally a relatively long incubation period before clinical signs appear, mostly dependent on the age, sex and physiological status of animals. As an example, animals infected during the critical periods of the pregnancy (about half of pregnancy) will develop clinical signs (*i.e.*, abortion) earlier (30-45 days after infection) than when pigs are infected out of the pregnancy period (*i.e.*, no abortion).

The *B. suis* entry sites are also similar to those identified in other *Brucella* spp. infections, being essentially the oral, nasopharyngeal, conjunctival and sexual mucosae. How *Brucella* spp. penetrate the epithelial lining of these mucosae, an essential event in pathogenesis, remains to be determined.

After penetration, a submucosal inflammatory reaction is produced. This reaction is characterised by infiltrates of mononuclear, polymorphonuclear and eosinophilic leucocytes. Invading brucellae are then addressed to regional lymph nodes by the lymphatic drainage. It is unclear if bacteria arrive to regional lymph nodes carried within phagocytic cells, as free extracellular organisms or in both ways.

Under experimental conditions, *B. suis* remains confined to the lymph nodes close to entry sites for 2 to 3 weeks. With the development of lymphadenitis close to these entry sites, *B. suis* reach blood via the efferent lymph, and bacteremia leads to a generalised infection in reticuloendothelial organs, lymph nodes distant from entry sites, genital and extragenital organs and accessory sexual glands.

B. suis can be isolated from liver, kidney, spleen, testes, epididimydes, vesicular glands, prostate, bulbourethral glands, uterus, mammary glands and most lymph nodes: submaxillary, parotid, retropharyngeal, prescapular, precrural, supramammary, and the ischiatic lymphocentre and lymph nodes draining to it. However, not all the infected animals excrete *B. suis*, and moreover, this excretion can be intermittent. Other organs such as the brain, vertebral column and synovial structures can be also found infected by *B. suis* in some animals. The involvement of joints and bones appears more important in pigs than in any other domestic species. Arthritis may occur in various joints, and sometimes spondylitis occurs.

The bacteria are not present in meat during the natural course of *Brucella* infections. Meat is not a target for any *Brucella* infection. It may be a consequence of contamination during slaughtering - carcass processing (*i.e.*, through contaminated milk or amniotic or allantoidal liquids). If present, the bacteria are only in the surface of the carcasses, and the risk of human contamination should be minimal. The presence of bacteria in the lymph nodes is also of little (if any) significance for transmission to humans.

4.2. Immune response

Due to complex and not fully understood virulence mechanisms, *B. suis* is able to survive and multiply inside phagocytic cells, but is also capable to invade a wide variety of cell types with the progress of infection. Initially, in the absence of antibody or complement mediated opsonisation, extracellular bacteria bind to lipid rafts and membrane receptors of macrophages. *B. suis* will survive during the entire life span of cells since *B. suis* infection does not induce apoptosis.

Like in most virulent brucellae, macrophages are the substrate for *B. suis* replication as well as the vehicles for spreading to different tissues and organs. With the progress of infection in the pregnant animals, erytrophagocytic trophoblasts act as replicating host cells and are the main site from which bacteria spread to foetal membranes and foetus.

The chronic infection results from the ability of *B. suis* to survive reactive oxygen intermediate and nitric oxide killing in host phagocytes, following which they activate bacterial genes in response to the acidic phagosome environment, preventing phagolysosomal fusion by remodeling the intracellular compartment, and subsequently replicating intracellularly. In these phagocytic cells, *B. suis* is able to colonise the endoplasmic reticulum where it multiplies actively. During this phase, *B. suis* is able to prevent apoptosis. A typical chronic inflammation is then established in the different organs colonised. As the chronic inflammatory response develops, cytokines, chemokines and other inflammatory mediators are released causing chronic to granulomatous inflammation with infiltrates of lymphocytes, macrophages, plasma cells and multinucleate giant cells, followed by necrosis, fibrosis and granulation tissue formation. The granulomas tend to undergo caseous necrosis and become encapsulated by connective tissue.

Infection of pigs with *B. suis* results in a chronic process that is usually nonlethal. The excreting pigs will continue to present a source of infection for non-infected pigs. One crucial component of immunity that results in survival of the host and the maintenance of this chronic infective state is gamma-interferon (IFN-γ), a cytokine of different T cell subsets. *B. suis* induces a strong immune response whose main components include the induction of T-cell cytokines such as IFN-γ, cytolytic activity by some T-cell subsets, and the production of specific antibodies. IFN-γ is considered the crucial effector cytokine for activating macrophages for efficient killing and inhibition of intracellular replication. Cytotoxic T-cells can theoretically prevent the sustained infection by killing infected host cells either by perforin-mediated cytolysis or other mechanisms.

Although these immune responses have been referred generally to as cell-mediated immunity, it may be more appropriate to refer to them as type 1 immunity as an abstraction from the original Th1 CD4 T-cells that produce IFN-γ. This is because not all T-cells participate in this part of immunity (*i.e.*, Th2 T-cells do not produce proinflammatory cytokines but rather those that promote production of antibodies)..Moreover, cellular immunity may require the existence of circulating antibodies able to promote phagocytosis. Immunological memory by

cells of the adaptive and, perhaps, bridging immune systems, meaning T lymphocytes and antibody-producing B lymphocytes, seems to be the keystone to effective immune responses. However, cells of the innate immune system may contribute also to controlling infections not only by their role as end-stage effector cells as well as by producing appropriate cytokines upon initial encounter with the pathogen leading the adaptive or acquired immune response to a type 1 pathway.

The *B. suis* surface S-LPS epitopes and other antigens are highly efficient at eliciting specific antibodies in infected swine. Moreover, *Brucella* LPS is a prototypical T-cell independent antigen because it can directly activate B-cells to produce antibody without the aid of helper T-cells. Antibodies have traditionally been considered to have a positive effect on protection against *Brucella* through their opsonic properties and their complement-mediated killing abilities, as well as agglutinate bacteria for clearance, mediate antibody-dependent cellular cytotoxicity, and by binding to bacterial receptors to prevent adherence of bacteria to host tissues.

The pattern of antibody production in pigs infected with *B. suis* has not been properly established. However, it should be similar to that induced by T-cell–independent antigens in the case of other *Brucella* infections. In these conditions, especific IgM anti-*Brucella* antibodies predominate in the first 2 weeks after infection, whereas the IgG isotypes increase slowly in the blood over the first 3 weeks of infection.

Opsonization is considered as the principal mechanism involved in protection by specific antibodies because it enhances phagocytic uptake of brucellae, which enhances intracellular killing in some cases. The relative contribution of IgG versus IgM antibody isotypes in brucellicidal functions of macrophages has not been evaluated. The role of antibodies with regard to complement-mediated killing mechanisms is questionable because some *Brucella* spp. strains are not susceptible to complement (Kirkbride, 1990; Dial *et al.*, 1992; Jubb *et al.*, 2007; Alton, 1990; Enright, 1990; Baldwin and Goenka, 2006; Moreno and Gorvel, 2004).

4.3. Vaccination

To date (June 2009), no fully safe and effective vaccines have been developed for *B. suis*.

The mechanisms conferring immunity against *Brucella* spp. in individuals are not yet completely understood. It is believed that circulating bactericidal (anti-LPS) antibodies are important to control the infection in the first stages of disease. After the opsonised bacteria have invaded the cells, phagocytic pathways are switched on to kill the bacteria, thereby preventing chronic infection (Schurig *et al.*, 2002).

The immune mechanisms dealing with protection are poorly understood since the infection can be established in presence of circulating antibodies, and on the contrary, in absence of antibodies, the infection can be avoided by the passive transfer of activated T-cells. For the use in pigs, only few vaccines have been developed or vaccine candidates have been studied in experimental trials.

Vaccines based on killed/inactivated brucellae or DNA / subunit vaccines do not protect from infection nor have yet been licensed for use in domestic pigs or wildlife so far.

Despite promising results in field trials (Lord *et al.*, 1997; Lord *et al.*, 1998; Edmonds *et al.*, 2001) *B. abortus* vaccine strain RB51 proved to confer no cross-protection against *Brucella* spp. in pigs (Moriyon *et al.*, 2004; Stoffregen *et al.*, 2006).

In China control of porcine brucellosis caused by *B. suis* biovar 1 (sporadically also by other *Brucella* spp.) was based on an attenuated smooth type *B. suis* biovar 1 strain, *i.e.* '*Brucella suis* S2' which was isolated from a pig foetus in 1952 in China. It was attenuated by serial cultivation (Deqiu *et al.*, 2002). It can be applied parenterally or *per os* (Xie, 1986). In the latter case the vaccine fluid should be sweetened with sugar syrup and mixed with beacons which may produce lesions in the mucous membrane of the snout thereby enhancing penetration of bacilli (Edmonds *et al.*, 2001). Pigs should be immunised twice with two doses of 20 x 10^{10} cells in an interval of 2 to 3 months. It might cause abortion in pregnant sows. Under field conditions it was possible to reduce the number of sero-positive animals from ca 70% to approx. 2% within a two years period, additionally applying rigorous test-and-slaughter and vaccination policy (Xie, 1986). S2 vaccine also induces antibodies which are believed to be non-persisting but cross-react with those resulting from natural infection, therefore interfering with serological routine diagnosis.

No live vaccine is available to protect single animals with 100% protection or without sporadic side effects (*i.e.* abortion in pregnant females and allergy). Thus, vaccination cannot protect pig holdings from sporadic infection or prevent shedding of the agent by single animals. Currently available vaccines produces anti-LPS-antibodies interfering in the interpretation of the serological tests results (used in the EU). The problem with the lack of test able to differentiate vaccinated from infected may hamper the control of *B. suis*. Live vaccines are still considered a risk for humans.

Conclusions

The pathogenesis of *B. suis* in swine has not yet been explained fully. There is generally a relatively long incubation period before clinical signs appear, mainly depending on the breeding status of the infected animals. Thus, the spread of infection with infected but apparently healthy animals is possible. *B. suis* remains confined to lymph nodes close to entry sites for 2 to 3 weeks, then bacteremia leads to a generalised infection including genital organs and accessory sexual glands.

B. suis can be excreted in vaginal excretions and milk of infected sows and semen in infected boars. This excretion seems to be the most relevant mechanism for *B. suis* spreading.

The immunological responses in pigs infected with *B. suis* have not been properly established. However, it is reasonable to expect that they would be similar to that induced by T-cell–independent antigens in other *Brucella* infections.

Currently available vaccines provoke anti-LPS-antibodies which interfere with serological tests used in the EU and may thus even contribute to the spread of the disease. Live vaccines still carry the risk of human infections.

To date (June 2009), no fully safe and effective vaccines have been developed for *B. suis*.

Recommendations

Although vaccination is considered as a control measure for the disease, currently available vaccines are not recommended for the control of porcine brucellosis caused by *Brucella suis* biovar 2 in Europe.

If suitable vaccines become available in the future, their use to control the disease should also be taken into consideration for wild boars or domestic pig breeds at risk of extinction (*e.g.*, the Iberian pigs).

5. Clinical signs and lesions of *B. suis* infection in swine

5.1. Acute and chronic brucellosis

Reproductive failure characterised by abortion, stillbirth and infertility in sows, testicular lesions, asymmetry of testicles and infertility in boars is the main clinical feature of *B. suis* infection. However, these clinical signs are not pathognomonic and several other pathogens can cause reproductive failure in swine. Potential aetiologies causing reproductive problems in pigs include *Actinobacillus* spp., *Streptococcus* spp., *Erysipelothrix* spp., *A. pyogenes*, *Pasteurella* spp., *Salmonella* spp., *Bacillus* spp., *Escherichia coli* and various other bacterial organisms, as well as several viral infections including pseudorabies (PRV), transmissible gastroenteritis (TGE), swine influenza (SIV), porcine reproductive and respiratory syndrome (PRRS), porcine parvovirus (PPV), enteroviruses (PEV) and encephalomyocarditis virus (EMCV).

Sows experimentally exposed to *B. suis* either before mating or during late pregnancy do not usually abort, and only sows exposed during early to mid pregnancy eventually abort. As the infection progresses, the bacteria become localised in the placenta and reach the foetus through chorion vessels. Infected sows develop several degrees of placentitis causing foetal malnutrition and hypoxia which results in abortion or premature or weak piglets, and then increasing perinatal mortality. Mummified foetuses have also been described. Abortion usually takes place from mid to late pregnancy, but there is a high incidence of stillborn and weak piglets and, as mentioned before, a high level of foetal resorption. Placental retention is also evident in a relevant proportion of infected sows. Fertility is reduced at herd level and many of infected sows repeat oestrus and remain fully non-productive. After abortion, the placenta may be edematous and hyperemic, and the foetus may have hemorrhagic fluid in the peritoneal space and subcutaneous tissues. The placenta may be retained. Metritis sometimes occurs, and nodules and abscesses may be found in both the gravid and non-gravid uterus. Lesions in the uterus are frequent sequelae after *B. suis* infection, being the main responsible of infertility. These have been referred to as miliary uterine brucellosis, and are characterised by the presence of many 2-3 mm pale yellow nodules seeded on the uterine mucosa, that can express a caseous exsudate when incised. When numerous, they tend to coalesce forming plaques and uterine thickening. Small reddish granulomas are often scattered over the uterine surface.

Orchitis, metritis and abortions have been observed as well in wild species but the frequency seems to be relatively low compared to the domestic species (S. Rossi, ONCFS France, personal communication, March 2009).

5.2. Macroscopic and microscopic lesions

Although the number of infected boars having palpable testicular alterations is not usually high, an important proportion of infected boars excrete *B. suis* in semen. After necropsy in boars, inflammatory lesions, abscesses or calcified foci may be seen in the testes and accessory sexual glands and organs, particularly the epididymis and seminal vesicles. In boars,

lesions tend to be unilateral. As evidenced in other *Brucella* spp. infections, testicular atrophy and a variable degree of enlargement of epididymis tail are characteristics of the chronic phase of the disease. Macroscopical appearance of testes is usually normal, but granulomas and calcification may be apparent on the cut surface. The affected epididymis appears firm, showing a white cut surface as a consequence of connective tissue proliferation. One or more abscesses resembling spermatoceles filled with creamy or caseous substances can be observed in the thick connective tissue. Haemorrhages and exsudative inflammation in the tunica vaginalis are frequent findings, and result from a rupture of the basic lesion (spermatocele) of epididymis. The organisation of these exudates leads to the formation of adhesions between the two layers of the tunica vaginalis. Vesicular glands can show enlargement and altered cut surfaces with dilated ducts, either empty or filled with fluid. No pathognomonic lesions have been observed in cases of *B. suis* infection in boars. Infected boars can have an impaired fertility, but do not necessarily show poor semen quality and lowered fertility.

Abscesses or other purulent lesions can also be found in non-reproductive organs, particularly the lymph nodes, spleen, liver, kidneys, joint capsules, tendon sheaths, bones, mammary gland, urinary bladder and, occasionally, the brain. Nodular splenitis, arthritis, bursitis and osteomyelitis of the vertebral bodies have also been reported. Swollen joints and tendon sheaths, accompanied by lameness and incoordination, can occur in both swine sexes. Viable *Brucella* may be present in these tissues. Less common signs include posterior paralysis, spondylitis and abscess formation in various organs. Although some pigs can recover from infection, most remain permanently infected. Some infected animals remain fully asymptomatic.

Several domestic species have been reported to be susceptible to *B. suis* infection. Horses exposed to infected pigs can also be infected, although this occurs rarely. *B. suis* usually causes inflammation of the supraspinous or supra-atlantal bursa in horses, this syndrome being known, respectively, as fistulous withers or poll evil. The bursal sac becomes distended by a clear, viscous, straw-colored exudate and develops a thickened wall. In chronic cases, nearby ligaments and the dorsal vertebral spines may become necrotic. *Brucella*-associated abortions have been reported rarely in horses. Infection has been also reported in dogs causing lameness and granulomatous lesions in genital organs. *B. suis* infection in cattle has been considered non-contagious and of little clinical relevance. However, the disease has been transmitted from infected pigs to cattle, causing infection of the udder and uterine tissues, with excretion of the microorganisms by milk and vaginal exudates.

It is very difficult to ascertain whether these species may act as a source of infection for pigs. According the preferred (natural) hosts for each *Brucella* species, these animal species are not the target host of *B. suis* infection. Then, they would not act as reservoirs of the infection for pigs and wild boars. However, in critical infection periods (i.e., gestation followed by abortion), these animals could contribute to the transmition of the infection.

In hares, infection by *B. suis* biovar 2 produces nodules in the internal organs, particularly the reproductive organs, as well as the subcutaneous tissues and muscles. The grey to yellowish nodules can become purulent. The animal's body condition may be minimally affected.

The lesions in wild boars are essentially the same as those described in domestic pigs. (Kirkbride, 1990; Dial *et al.*, 1992; Jubb *et al.*, 2007; Alton, 1990; Enright, 1990; Moreno and Gorvel, 2004).

Conclusions

Reproductive failure characterised by abortion, stillbirth and infertility in sows and testicular lesions and infertility in boars is the main clinical feature of both acute and chronic infection due to *B. suis* biovar 2 in pigs. Although some animals can recover from infection, most of them will remain permanently infected.

Several domestic species including cattle, goats, horses, and dogs have been found infected and showing clinical signs, but these domestic species have been considered of little or no relevance at all to the epidemiology and transmission of infection to pigs (accidental hosts). In hares, infection by *B. suis* biovar 2 produces also gross pathological lesions, but in some cases the body condition is minimally affected.

6. Diagnosis of *B. suis* infection in swine

As previously described, Brucellosis is not more pathognomonic in swine that it is in ruminants and diagnosis depends on the interpretation of both, field (epidemiological and clinical) and laboratory investigations.

6.1. Tests available

Unequivocal diagnosis of *B. suis* infections can be made only by the isolation and identification of *Brucella*, but in situations where bacteriological examination is not practicable, diagnosis can be based on immunological methods (identifying the immunological response of the host towards *Brucella* infection). Methods and tests used for the diagnosis of porcine brucellosis are very similar or identical to those applied for the diagnosis of brucellosis in cattle and small ruminants. Refer to the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2008a) for a detailed description in details of the available tests.

6.1.1. Direct diagnosis

As in ruminants brucellosis, the presumptive diagnosis of brucellosis in pigs can be made by the microscopic examination of Stamp's stained smears from vaginal swabs, placentas, aborted foetuses or lymph nodes. With regard to morphological staining characteristics, *B. suis* is indistinguishable from other smooth *Brucella* spp. However, this test lacks sensitivity and specificity, and isolation of *Brucella* on appropriate culture media allows a more accurate diagnosis.

For the diagnosis of brucellosis by cultural examination, the choice of samples usually depends on the clinical signs observed.

The most valuable samples from living animals include aborted foetuses or dead piglets (stomach contents, spleen and lung), foetal membranes, vaginal secretions (swabs), milk, semen and arthritis or hygroma fluids.

From animal carcasses, the preferred tissues for culture are those of the reticulo-endothelial system (*i.e.* head, mammary and genital lymph nodes and spleen), the late pregnant or early post-parturient uterus, and the udder. As reported before, *B. suis* can also be isolated from liver, kidney, testes, epididymides, vesicular glands, prostate and bulbourethral glands.

B. suis grows well on the usual *Brucella* media without the addition of serum or enrichment of the atmosphere with carbon dioxide. However, since brucellosis in pigs could also be due to *B. abortus*, in areas where this species is highly prevalent in cattle, it is recommended in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2008a) to incubate culture plates also at 37 °C in air supplemented with 5–10% (v/v) CO_2.

B. suis is generally resistant to the antibiotics used to prepare selective media for the culture of *Brucella*. However, as mentioned before, the biovar 2 of *B. suis* appears to be highly sensitive to selective media and could be more difficult to isolate than biovars 1 and 3. Therefore, the sensitivity of culture increases significantly by the simultaneous use of both Farrell's and the modified Thayer–Martin's medium (Marin *et al.*, 1996). As far as the biovar 2 is concerned, the simultaneous use of non-selective media is also recommended (Garin-Bastuji and Blasco, personal communication, March 2009).

Growth normally appears after 3–4 days, but cultures should not be discarded as negative until 8–10 days have elapsed. *B. suis* colonies are morphologically indistinguishable from other smooth brucellae and can be presumptively identified as *B. suis* by agglutination with monospecific antisera. The three most important biovars involved (1, 2 and 3) agglutinate with the A but not the M monospecific antisera (Alton *et al.*, 1988; OIE, 2008a).

In addition, species and biovar identification can be accomplished by routine typing tests such as production of hydrogen sulphide, growth in the presence of dyes, phage typing and oxidative metabolic tests. Additional tests such as the urease reaction and inhibition by safranin may be useful. It should be noted that some biovar 1 strains may be atypical in being resistant to basic fuchsin. However, biovar 1 is the only *B. suis* biovar to produce hydrogen sulphide. Similarly, the strains identified as biovar 3 by conventional biotyping and isolated up to now in Europe (Croatia), while not producing hydrogen sulphide, are classified as biovar 1 by all molecular tools available up to now. The main differential characteristics are described in Table 1.

Table 1. Differential characteristics of *B. suis* biovars 1, 2 and 3

			Dye tests		**Agglutination with monospecific sera**		**Lysis by phage**		
Biovar	Requirement for CO_2	H_2S production	Thionine	Basic Fuchsin	A	M	Tb RTD	Tb 10^4 x RTD	R/C RTD
B. suis 1	-	+	+	-	+	-	-	+	-
B. suis 2	-	-	+	-	+	-	-	+	-
B. suis 3	-	-	+	+	+	-	-	+	-

Molecular genetic techniques using the Polymerase Chain Reaction (PCR) and specific primers are available, allowing the adequate identification of *B. suis* and other species of *Brucella.*

Molecular biology has made a valuable contribution by greatly reducing diagnosis times and improving accuracy of results (Whatmore *et al.*, 2005). These molecular methods include PCR, Restriction Fragment Length Polymorphism (RFLP), Variable Number of Tandem Repeats (VNTR) (Kattar *et al.*, 2008), northern blots, sequencing of complementary DNA (cDNA) libraries, serial analysis of gene expression (SAGE), microarrays including cDNA

(allowing the study of gene expression comparison in the particular tissue or condition) and oligonucleotide arrays for Microbial Diagnostic Micro-arrays (MDMs) (Duggan *et al.*, 1999).

To date (2009) the complete genomes of the following *Brucella* species are available: *B. abortus* biovar 1, 9-941; *B. abortus* biovar 1, 2308; *B. canis*, ATCC 23365, *B. melitensis* biovar 1, 16M; *B. melitensis* ATCC 23457; *B. ovis*, ATCC25840; *B. suis* biovar 1, 1330; *B. suis* biovar 2, ATCC 23445; and the vaccinal strain *B. abortus* S19 (Liolios *et al.*, 2006[10]). In January 2009 complete genomes of 19 other *Brucella* species were made available to the scientific community at the Broad Institute; their analysis and annotation is presently being studied by Virginia Bioinformatics Institute (VBI) and PathoSystems Resource Integration Centre (PATRIC).

It is expected that future genome sequencing of the *Brucella* spp. group would provide a better molecular understanding of human disease processes. Genome sequence information along with functional genomic tools of microarrays, RNA interference, gene transfection and other tools are front-line research tools (Simpson, 2002). This set of tools provides the basis for detailed understanding of the phylogenetic relationships and evolution. It is also providing novel insights into the function of individual genes (Simpson, 2002). With the event of new genomic tools, new molecular targets will be identified and tested and unique patterns will be associated with each of the sequenced *Brucella* spp. The PATRIC database already allows to find groups of unique orthologous genes for the *Brucella* species studied, all of the *Brucella* spp. sequenced and studied have some unique signature sequences, as well as *B. suis*.

Despite the high degree of DNA homology within the genus *Brucella*, several molecular methods, including PCR, PCR RFLP and Southern blot, have been developed to allow, to a certain extent, differentiation between *Brucella* species and some of their biovars (Bricker, 2002).

A new multiplex PCR assay (Bruce-ladder) has been proposed for rapid and simple one-step identification of *Brucella* (López-Goñi *et al.*, 2008). In contrast to other PCRs, that cannot differentiate the different biovars of *B. melitensis* and *B. suis* and can differentiate only biovars 1, 2 and 4 of *B. abortus*, Bruce-ladder is also able to identify *B. abortus* biovars 3, 5, 6, 7, 9, and *B. suis* biovars 2, 3, 4, 5 and to detect DNA from *B. neotomae*, *B. pinnipedialis* and *B. ceti*. Its only inconvenience is that some *B. canis* strains can be identified erroneously as *B. suis.*

Alternative approaches allowing identification of all *Brucella* species based on Single Nucleotide Polymorphism (SNP) discrimination by either primer extension or real-time PCR have been described (Gopaul *et al.*, 2008). These tests are rapid, simple and unambiguous and, being based on a robust phylogenetic analysis, overcome some problems seen with Bruce-ladder such as the misidentification of some *B. canis* isolates. Nevertheless, their use is restricted, to well-equipped and experienced laboratories.

Other methods have been described recently that include multilocus sequencing (Whatmore *et al.*, 2007) and several typing schemes based on the use of Multiple Locus Variable number of tandem repeats Analysis (MLVA; Le Flèche *et al.*, 2008). Depending on the particular markers chosen, these methods allow isolates to be differentiated to the species level or to be further subdivided at the infra-species level, providing additional epidemiological information.

[10] http://www.genomesonline.org/gold.cgi?want=Published+Complete+Genomes accessed on June 2009.

6.1.2. Indirect diagnosis

The major antigen involved in the immunological response of the swine host and in serological tests currently available is the S-LPS. As mentioned before, the OPS moiety of this molecule contains epitopes that cross-react with those existing in the corresponding S-LPS of *Y. enterocolitica* O:9 and no available serological tests based on this antigen are able to distinguish between antibodies raised to these two infections. *Y. enterocolitica* O:9 infection in pigs is apparently common in some EU areas and, accordingly, this represents a major complication for the diagnosis of *B. suis*.

Swine serum may sometimes also contain nonspecific antibody, thought to be of the IgM isotype, further reducing the specificity of conventional tests, especially the serum agglutination test (SAT).

Conversely, as mentioned before, the effect of the infection in pigs is more variable among individuals than in any other domestic species. Also, swine complement interacts with guinea-pig complement to produce a pro-complementary activity that reduces the sensitivity of the CFT.

The only available allergic skin test is based on the use of Brucellin that is a S-LPS-free cytosolic extract from rough *B. melitensis* strain B115. This preparation does not stimulate the formation of antibodies that would be reactive in RBT, CFT or ELISAs. It has been developed for use in ruminants, but it is also effective for confirming the disease at the herd level in pigs. The brucellin is not being currently produced commercially.

Thus, the immunological diagnosis of porcine brucellosis is quite difficult and, moreover, it has been suggested that the performances of the different tests can be expected to vary under various epidemiological situations (Garin-Bastuji *et al.*, 2008). Available data on the diagnostic performance of serological and skin tests have been systematically assessed and summarised (Chapter 7).

6.2. General scope of tests

There is relatively little information on the value of the different serological and/or allergic skin tests in either free or infected populations in field conditions.

Several studies have suggested that the sensitivity and specificity of the RBT, the indirect (iELISA) and competitive enzyme-linked immunosorbent assay (cELISA), and the fluorescent polarisation assay (FPA), are similar for the diagnosis of *B. suis* infection.

However, important differences in the sensitivity/specificity ratios of the serological tests for *B. suis* have been reported, according the validation criteria and the different epidemiological conditions used.

In some situations, the use of the FPA or cELISA has been reported to reduce cross-reactivity with *Y. enterocolitica* but this should be confirmed in additional field studies performed in various epidemiological situations. Sensitivity levels may be low for the CFT, and this low sensitivity can be even lower considering the relatively high percentage of sera showing anticomplementary activity. Therefore, caution should be taken when interpreting test results from individual animals.

While RBT and CFT are standardised against the OIE International standard serum (OIEISS) for use in pigs, up to now the conditions for standardizing ELISAs and FPA in pigs have not been defined due to the absence of an internationally recognised porcine standard serum,

using the validation criteria recommended by the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2008a). Primary reference standards sera are currently being developed and will be available to NRL when completed (OIE, 2008a).

For international trade and intra-Community movement of boars or semen donors, the disease status of the herd of origin and of the area in which the herd is situated may be classified as free of *B. suis* more easily than the same status for individual animals. However, in case of small herds of origin (*e.g.*, less than 50 animals), the herd-level sensitivity equals the animal-level sensitivity (Greiner and Dekker, 2005).

Since bacteriology and allergic skin test with Brucellin, are not based on cross-reactive antigens, the only confirmatory tests theoretically suitable to fully discriminating between true brucellosis infections and the infections caused by *Y. enterocolitica* O:9 or other cross-reacting bacteria.

Conclusions

Despite their respective failures in sensitivity/specificity, and even considering that some of the serological tests have not been fully standardised for use in pigs, almost all of currently available serological tests (*i.e.* RBT, CFT, ELISAs and FPA) are sensitive enough as screening tests to detect problem herds. The presence of a large proportion of animals positive in different tests is also an indicator of high value for suspecting brucellosis in a herd with evocative clinical signs (infertility, abortions, orchitis and/or arthritis).

Molecular techniques are currently available for identifying *B. suis* and other *Brucella* species. Some of these techniques can also distinguish biovars of *B. suis*. Depending on the particular markers chosen, these methods allow isolates to be differentiated to the species level or to be further subdivided at the infra-species level, providing additional epidemiological information.

All current serological tests using the S-LPS (e.g. BPAT, RBT, SAT, CFT, and iELISA) or the O-PS (e.g. cELISA, some iELISA and FPA), cannot fully differentiate serological responses caused by brucellosis infection from those caused by Yersinia enterocolitica O:9 or other bacterial infection that can cross-react with these species. The reason for this cross-reaction is due to the fact that the antigens used for these tests share important and extensive common epitopes with these bacteria species.

Therefore, in herds in which the presence of *Brucella* has not been yet confirmed by isolation of the bacteria, all positive reactions obtained in the S-LPS or O-PS based tests should be in principle investigated with the aim to exclude the possibility of a cross-reaction (i.e. False Positive Serological Reaction, FPSR).

The brucellin skin test, however, uses cytosolic proteins as antigen, and these have been proven as highly specific for the genus *Brucella* species (with the only exception of Ochrobactrum intermedium. However, it is not considered to have a relevant occurrence in pig populations). Furthermore, the brucellin skin test measures the cell mediated immune response and not humoral response. The brucellin skin test can therefore be considered as a candidate test for the purpose of confirming the *Brucella* infection in a pig herd after a positive serological result.

Recommendations

The isolation (or molecular detection/identification) of the strain involved in *B. suis* outbreaks should be attempted, whenever possible, because it is frequently the only mean for confirming the infection. Moreover, this microbiological investigation is also required for epidemiological studies and to verify whether *B. suis* biovars 1 or 3 or other *Brucella* species (like *B. abortus* or *B. melitensis*) could have been introduced in the EU pig population.

It is recommended to carry out further studies to assess the use of existing tests (such as PCR and Culture) for the diagnosis of *B. suis* in semen.

Further development of Brucellin-based tests should be encouraged since, in addition to bacteriology and molecular tools, these tests are the only confirmatory tests suitable to fully discriminate between true brucellosis infections and infections caused by *Y. enterocolitica* O:9 or other cross-reacting bacteria. This development should include the standardisation of the antigen and of the application and reading of test results, the validation of the diagnostic sensitivity and specificity (with and without concurrent *Y. enterocolitica* O:9 infection) and the availability of Brucellin standard preparations and application protocol.

It is recommended to develop a common database for the strains of *B. suis* isolated in EU to be used to support future epidemiological investigation.

7. Meta-analysis of Sensitivity and Specificity of diagnosis tests for Porcine brucellosis

Within the framework of this report, efforts were made to create and analyse a comprehensive and unbiased basis for assessing the available scientific evidence on the validation status of diagnostic tests for *B. suis* infection in pigs. The target parameters for this task were the diagnostic sensitivity (Se, the probability of correct positive test result in infected pigs) and specificity (Sp, the probability of correct negative results in non-infected pigs). Bacteriological culture was considered as one criterion for establishing the true infection status in individual animals for estimation of Se (reference diagnostic or gold standard). As a consequence of using bacteriological culture for definition of the reference status, no estimates of Se and Sp for bacteriological culture will be available. However, efforts were made to identify any study that would present cross-tabulated results of several tests including culture, which would allow the estimation of the diagnostic performance of all tests including culture by latent class analysis. Sources of information were a) the published scientific literature assessed by systematic review, b) a questionnaire sent to EU National Reference Laboratories (NRL) and c) a questionnaire sent to associations of and individual veterinary diagnostic companies (see Appendix 3 for details). The test results pertaining to pigs from officially free areas as obtained from NRLs (Appendix 4) suggest that false positive serological reactions (FPSR) were not reported by all NRLs except for the iELISA. Preliminary analyses of the iELISA based on data sources other than NRL demonstrated almost perfect Sp. Therefore, only the NRL data for iELISA pertaining to pigs from officially brucellosis free areas were included in the meta-analysis. All pertinent information related to Se and Sp estimates and meta-data (*e.g.*, description of the tests and study populations) were summarised in data collection sheets (see Appendix 5). The goal of this task was to establish statistical summary estimates of Se and Sp which account for the choice of the reference method and study design (epidemiological study versus experimental study). Consistent exclusion and inclusion criteria were applied to all three sources of information (Appendix 6).

7.1. Systematic review and analysis

The working group has conducted a systematic review on the use of diagnostic tests for *B. suis*. Using a reference list provided by diagnostic experts in the group, the following search string was developed such that all known relevant articles were captured and the number of irrelevant articles was minimised.

> (brucel*) AND (suis OR porc* OR pig* OR swine OR sow* OR boar* or hare*) AND (test* OR diagn* OR lymph* OR rbt OR (rose AND bengal AND test) OR cft OR (complement AND fixation AND test) OR bbat* OR (buffered AND brucella AND agglutination AND test) OR bpat* OR (buffered AND plate AND agglutination AND test) OR fpa OR (fluoresce* AND polari* AND assay*) OR elisa OR pcr OR skin* OR allerg* OR hypersens* OR SAT OR (Serum AND agglutination AND test)) AND (sens* OR spec* or accura* OR perfor* OR eval* OR valid* OR detect*)

The search was run in ISI web of knowledge (www.isiknowledge.com). Neither publication date nor language was used as exclusion criterion. The review process was organised in two stages involving six reviewers, who were also members of the Working group . Each paper was allocated randomly to two reviewers. In the first stage, only title and abstract was used to select an article for full review. Only those papers were excluded where both reviewers independently voted for exclusion. In the second stage, the papers were reviewed sequentially. The reviewer randomly allocated as "first reviewer" for a given paper completed the review and filled a template (See Appendix 5). The data collected included the bibliographic information, information on the diagnostic tests evaluated, reference populations used and study results as well as inclusion/exclusion codes for paper, tests reference populations and results, comments and workflow checkboxes. The filled template was sent to the allocated "second reviewer", who was in charge to confirm all entries or discuss and consolidate any divergences with the first reviewer. In cases of unresolved discrepancies (which did not occur) the working group was in charge of final assessment. The workflow is shown in Appendix 7, Figure 22, and was organised using Microsoft® -Word form templates, read-out as text files and processed and analysed using R (R Development Core Team, 2009) and code generated for this purpose (available on request from the authors of the report). The scientific publications retained for the final analysis are listed in Appendix 8.

Specificity data from National Reference Laboratorie:

Results from the NRL of EU-27 MS were obtained within the framework of their national activities as regards Porcine Brucellosis and are summarized in Appendix 4. The data suggest not all MS reported the results including False Positive Serological Reactions (FPSR), which is required for estimating the Sp.

7.1.1. Statistical analyses (meta-analysis)

The statistical approach was essentially as described in previous EFSA reports involving a meta-analysis of diagnostic sensitivity and specificity (EFSA, 2006b; EFSA, 2008). The point estimates and exact binomial 95% intervals (using R function "binom.test") of all available estimates for Se and Sp were generated an plotted for exploration of the variability in the data (see Forrest Diagrames in Figure 1 and Figure 2). To explore publication bias, the arcsine-transformed Se and Sp estimates were plotted against the respective sample sizes (see funnel plot in Appendix 9, Figure 23 and Appendix 9, Figure 24). The transformation was chosen to achieve better approximation to Normal distribution. A lack of symmetry and in particular absence of low estimates from studies with small sample sizes can be interpreted as an

indication of publication bias. The summary analysis for Se for each of the candidate test consisted of a logistic regression model of the form

$$\text{logit Se} = a + b*X$$

where Se is the empirical sensitivity (number of true positives / sample size for Se), X is an indicator variable summarising the relevant study design features and a and b are estimates of the model coefficients using all available data for the given test. Intermediate results (if any) where considered as false negative test outcomes for calculating Se. For analysis of Se we defined X=0 as indicating the preferred study design, *i.e.* if the gold standard included the use of bacteriology and an epidemiological study type rather than experimental results was used and X=1 else, while for analysis of Sp only the study type criterion was used. Using the inverse logit link, we estimate the summary (meta-analytical) Se_{MA} for each tests and for the preferred study design (X=0) in terms of the single parameter a as

$$Se_{MA} = f(a) = 1/(1+\exp(-a)).$$

These models were setup separately for each of the available diagnostic tests to generate MA estimates of the Se for each of them. Two model estimation techniques were used. First, maximum likelihood estimation, implemented using R's "glm" function, was used for the purpose of investigating the impact of each of the papers on the summary estimate for Se. The principle is that for each diagnostic test, Se_{MA} was evaluated n times, where n is the number of papers contributing Se data for the given test is taken as an indication of the impact ("leverage") of each paper on the overall results for the tests. If only a single source paper provided data about Se of a given test, this paper was interpreted to have very high impact and the corresponding leverage value for this combination (paper, test) was set to the maximum observed value for any other combination. The results were plotted to allow visual identification of any paper with outstanding impact on the Se and/or Sp summary estimate (see "leverage" plots in Appendix 8, Figure 25 and Appendix 8, Figure 26). For example, RefID 1024 had a marked impact on the estimation of Se for BPAT, CFT and SAT for which also other papers contributed data. In contrast RefID 139 and 382 had a marked impact on estimation of Se for Lateral Flow Assay (LFA) and Rivanol test, respectively, whereby no other paper contributed data to those. The method was applied mainly for explorative purpose but also addressed concerns that high impact of individual papers could be due to data collection mistakes. The final results from logistic regression were obtained by fitting the models described above using Markov Chain Monte Carlo (MCMC) method (BRugs package, Andrew *et al.*, 2006) as described elsewhere (EFSA, 2006b; EFSA, 2008) (3 chains, burn-in 1000, 20,000 iterations, convergence monitored). As a possible approach to account for potential underlying variability of the Se parameter among the individual estimates the introduction of a random effect term for the intercept of the models was considered. However, for most diagnostic tests, the random effect term could not readily be estimated due to the limited number of available studies (results not shown) and therefore the final results were obtained from models without random effect. The model is shown in Appendix 9. The advantage of MCMC is that the posterior distribution of the parameter (a) can be used to obtain the distribution of Se_{MA}. The latter can be used instead of point values to capture statistical uncertainty for further stochastic modelling. Non-informative priors were used for the model parameters a and b. All analyses for Sp proceeded analogously. Consistently with the analysis of sensitivity, intermediate results (if any) where considered as true negative test outcomes for calculating the Sp.

7.1.2. Results of the meta-analysis

A total of 111 publications, five dossiers submitted from commercial companies and submissions of results from brucella-free areas from eleven EU MS were reviewed based on full text information (stage 2 of the review). A total of 18 of these papers/dossiers where found eligible according to the inclusion criteria, including from commercial companies (RefID 200901, 200902, 200903, 200904) and NRL Poland (RefID 2009001). The data from NRL France have also been submitted via RefID 200903. The scientific publications retained for the final analysis are listed in Appendix 8. For analysis of Se, 10 diagnostic tests could be evaluated based on a total of 38 estimates distributed across 12 source papers. For analysis of Sp, 11 diagnostic tests could be evaluated based on a total of 61 estimates distributed across 14 source papers. No studies could be identified that allowed the estimation of Se and Sp of bacteriological culture using latent class analysis. The Forrest plot for Se shows marked variability among estimates (Figure 1). The estimates for Sp are more uniform (Figure 2). The funnel plots were not indicative for the presence of publication bias for Se (Appendix 9, Figure 23) and Sp (Appendix 9, Figure 24) in which case low parameter estimates (towards the left side of the x-axis) would be expected to occur less frequently.

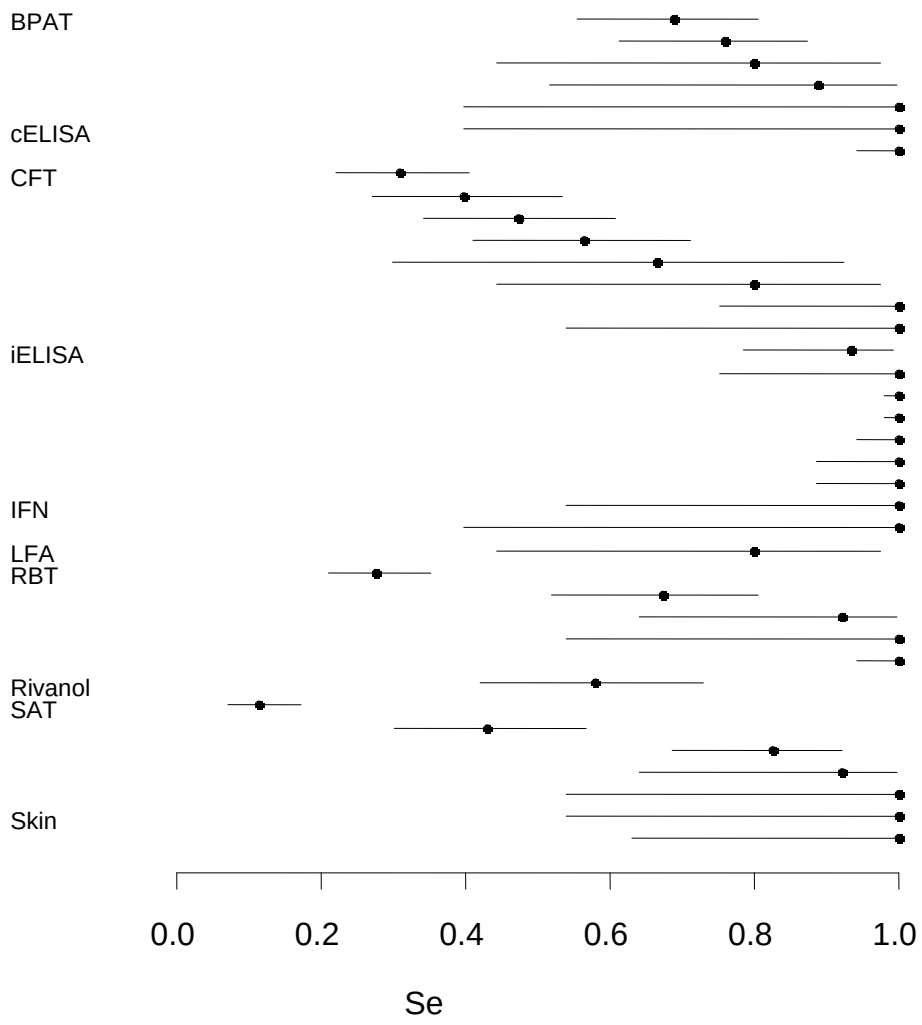

Figure 1. Forrest plot with point estimates for sensitivity (Se) and 95% confidence intervals for diagnostic tests for *B. suis* detection in pigs*.

*each available estimate is plotted.

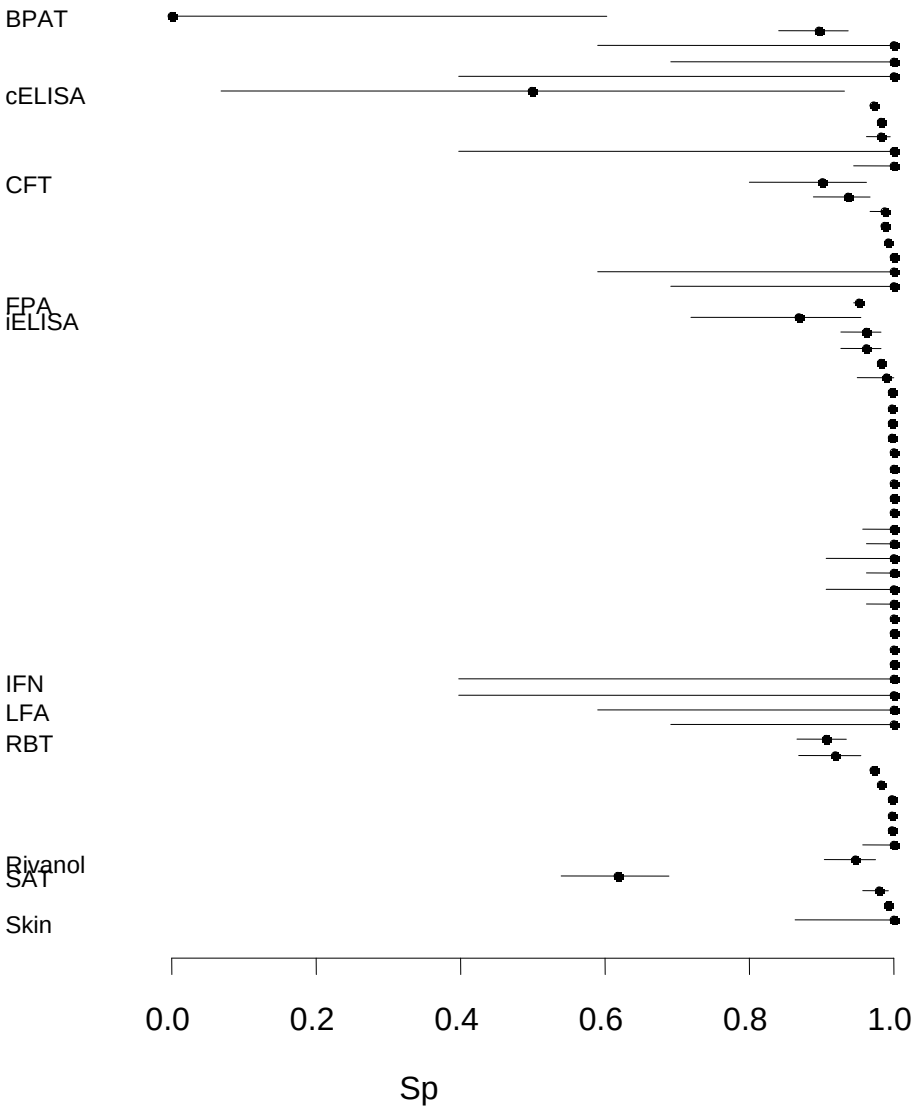

Figure 2. Forrest plot with point estimates for specificity (Sp) and 95% confidence intervals for diagnostic tests for *B. suis* detection in pigs.

The summary meta-analysis results for Se and Sp are shown below (Table 2 and Table 3) and will be used for simulation of the classification performance when used in the context of testing boars for admission to and during their stay on semen collection centres.

Table 2. Point estimates and lower limit (LL) and upper limit (UL) of 95% credible interval for sensitivity of diagnostic tests for *B.suis* based on meta-analysis (MA) of primary studies.

Test	MA Estimate	LL	UL	Number of primary estimates
BPAT	0.741	0.658	0.813	5
cELISA	1.000	0.988	1.000	2
CFT	0.533	0.464	0.602	8
iELISA	1.000	0.998	1.000	7
IFN	0.999	0.000	1.000	2
LFA	0.800	0.444	0.975	1
RBT	0.870	0.802	0.922	5
Rivanol	0.581	0.421	0.730	1
SAT	0.642	0.552	0.724	5
Skin	0.999	0.000	1.000	2

Note: If only one primary estimate is available, LL and UL refer to the lower and upper limit of the exact binomial 95% confidence interval, respectively.

Table 3. Point estimates and lower (LL) and upper limit (UL) of 95% credible interval for specificity of diagnostic tests for *B.suis* based on meta-analysis (MA) of primary studies.

Test	MA Estimate	LL	UL	Number of primary estimates
BPAT	0.908	0.861	0.943	5
cELISA	0.979	0.976	0.982	6
CFT	0.996	0.996	0.997	8
FPA	0.952	0.945	0.958	1
iELISA	0.999	0.999	1.000	24
IFN	0.999	0.000	1.000	2
LFA	1.000	0.949	1.000	2
RBT	0.998	0.997	0.998	8
Rivanol	0.949	0.905	0.976	1
SAT	0.986	0.983	0.988	3
Skin	1.000	0.863	1.000	1

Note: If only one primary estimate is available, LL and UL refer to the lower and upper limit of the exact binomial 95% confidence interval, respectively.

7.2. Suitability of available tests for porcine brucellosis

Using the results of the diagnostic Se and Sp from the systematic literature review, we further investigate the performance characteristics of these tests when using in parallel or sequential on individual animals under the assumption of conditional independence using formulas described by Gardner *et al.* (2000). The parallel scheme implies that two tests are used for the same individual animal, which is classified as "positive" if at least one test is positive and otherwise as "negative". The sequential scheme in this context implies that an animal is classified "negative" if the first test is "negative" (in which case no further test is applied) or if the first test is "positive" and the second (confirmatory) test is "negative". Final classifications as "positive" require that both the first and the second test give "positive" results. The specific context in which the use of diagnostic tests is considered, is the admission of boars to approved semen collection centres and the compulsory routine testing during the stay of boars

in or exit from centres (Chapter 9). Criteria for selecting candidate tests for further evaluation of their combined performance include the point estimates for Se and Sp, the strength of evidence (judged by number of estimates available and width of 95% credible interval), logistic (sampling and laboratory facilities) and immunological considerations. For example, the skin test would appear a suitable confirmatory test because its antigen is not related to the antigens of other tests. Generally, tests should be biologically independent to minimise risk for error correlation. However, the Brucellin antigen is currently not available (see Chapter 6) and also the database for Se and Sp estimation of the skin test is relatively poor so that this test was not included in further analyses. Other combinations of tests not listed below may be suitable as well and should be assessed using a similar approach.

It should be considered that, in most of the papers included in the Meta-analysis, the Sp results for iELISA were obtained in almost "ideal" conditions, using for instance sera taken from brucellosis-free pig herds and with no history of exposure with *Y. enterocolitica* O:9 (or even in some cases sera from Specific Pathogen Free [SPF] holdings), with the aim to establish the test cut-off resulting in the best diagnostic performance. This situation may explain why Sp in iELISA resulted in almost 100%, a situation which may be not representative of the real situation in the field.

For this reason, and only for the calculation of iELISA Sp, field data provided by either commercial companies and NRLs were considered in the analysis, whenever data presented were collected from certified brucellosis free MS or Region of MS.

Concerning the interpretation of "intermediate" results of iELISA tests, all results which were presented as not positive have been considered as test negative outcome for the purpose of the statistical analysis.

Conclusions

Antigens other than the S-LPS have been used with the objective of developing more specific tests, but, subsequently, up to now all the developed alternative tests lack appropriate sensitivity/specificity ratios.

Highly sensitive and reasonable specific testing systems with the potential to combine more than one test are required for a rigorous detection and slaughter policy. Evidence from our systematic review suggests that iELISA could be a suitable candidate because of its high Se and Sp, the latter being close to 100%. The performance of this test will be estimated in the explicit context of protocols for admission of boars to semen collection centres.

Currently, in pigs serological testing is only useful to monitor the status of a herd but not of single animals. Application of PCR for field use has to be standardised but still lacks sensitivity. This also applies to culture results using standard bacteriological procedures.

Recommendations

Formal procedures such as those implemented by the OIE should be considered for accreditation of candidate tests (*e.g.* iELISA) for the purpose of control of *B. suis* in pigs.

8. Factors associated with the introduction and spreading of porcine brucellosis in pig herds

This Section addresses the specific question on the risk of porcine brucellosis (*B. suis*) being present and introduced into domestic pigs, in particular through contact with wildlife, and subsequent spread within the EU by trade in pigs and pig semen. It identifies and qualitatively assesses the risk factors (RF) for such introduction and spread of *B. suis* infection on the basis of available evidence and uncertainty.

Therefore, the main objectives of this section are:

- To identify potential RF associated with epidemiological situations, management practices or measures that may modify the likelihood of a *B. suis* infection to become established in a domestic pig holding.
- To describe the role of RF in specified relevant pathways for trade-related *B. suis* risk.
- To qualitatively assess the level of occurrence of RF taking into account the (assumed) variability within and among MS.
- To qualitatively assess the adverse effect of the RF in terms of likelihood of *B. suis* infection becoming established in a domestic pig holding taking into account the (assumed) variability within and among MS.
- To describe and compare RF in terms of their level of occurrence and adverse effects as well as by their role in specified pathways.
- To account for the uncertainty of the qualitative scores as evidenced by differences among experts in their assessment.

The following sections provide a case definition of *Brucella suis* infection in pigs (Section 8.1), a conceptual framework for assessing risk factors (Section 8.2), a general description of each identified risk factor (Section 8.3), the description of the approach for (Section 8.4) and results (Section 8.5) of the qualitative assessment of risk factors.

8.1. Case definition of *Brucella suis* infection in pigs

For the purpose of this Opinion, the following case definition of *B. suis* applies to any domestic or wild pig animal (*Sus scrofa*):

- from which *B. suis* has been isolated, or
- for which the results of official serological tests[11], and
  - the presence of clinical signs such as abortion, or
  - the existence of epidemiological conditions concerning the animal, the herd or the territory of concern

  might indicate that the *B. suis* infection has occurred.

[11] To be specified in relation to this species.

8.2. Conceptual Framework

The goal of this section is a qualitative assessment of potential risk factors for *B. suis* infection becoming established in a domestic pig holding as this would present a sanitary threat for intra-Community trade. For the purpose of the qualitative assessment, a combination of the likelihood of occurrence of the risk factor and the magnitude of its consequences has been considered. As adverse effect, the impact is defined in terms of a likelihood of *B. suis* infection becoming established in a domestic pig holding. This section broadly follows the methodology for risk assessment as defined by the World Organisation for Animal Health (OIE, 2008b). For the purpose of this assessment, *B. suis* (biovar 2) has been identified as a hazard of concern. The following sections will describe the relevant pathways, and will provide a generic description the risk factors (Chapter 8.3), which will be qualitatively assessed (Chapter 8.4)

Based on the known transmission routes for *B. suis* (see Chapter 3) several pathways may need to be considered.

We consider a risk pathway as a sequence of events that may lead to introduction and dissemination of *B. suis*. For each of these pathways a specific set of events may need to come together at one point in time to result in the local introduction of the infection, or a wider subsequent dissemination within the EU following the introduction (Figure 3).

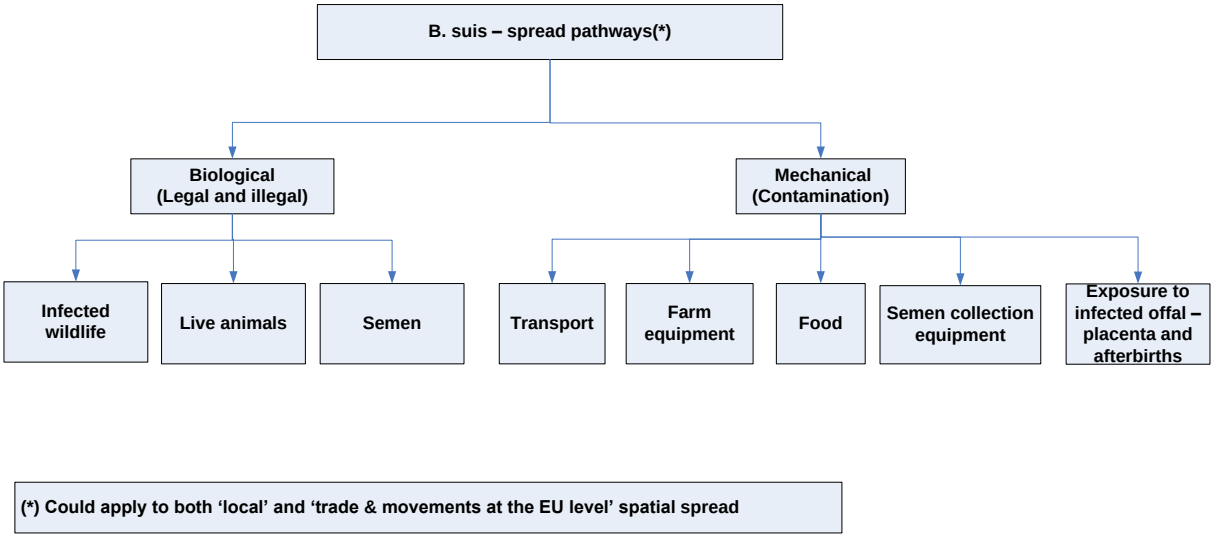

Figure 3. Biological and mechanical modes of introduction of *B. suis* into domestic pig holdings

Available evidence suggests that wildlife (WL, including wild boar and hares) and outdoor pig holdings may play a key role in the epidemiology of *B. suis*. Therefore, as a starting point, the hypothetical pathways need to consider the presence/absence of potentially infected WL

and/or outdoor pig holdings and the role that they may play in further dissemination of *B. suis* infection. In general terms of intra-Community trade, movement of potentially infected wildlife for hunting purposes, live pigs or semen is relevant when assessing the risks of further dissemination of *B. suis*.

It is important to note that while the presence of *B. suis* has been reported in wild boar and hares in some MS for decades, only a limited number of sporadic cases have been reported in outdoor pig holdings in some MS in the recent past. There is no available official or published data that would suggest that *B. suis* is currently present in any of the indoor commercial pig holdings in the EU.

On the other hand, this section acknowledges that transportation and movement of other biological materials, such as meat, meat products and miscellaneous related commodities may theoretically pose a trade risk. However, there is no evidence to support a relevant role of these commodities in the introduction of *B. suis* infection into commercial pig holdings or further spread within the EU (See Chapter 3).

Mechanical transmission may be relevant for local dissemination without direct impact on intra-Community trade. However, local dissemination could indirectly result in potentially increased intra-Community trade related risks if the infection is introduced in wider local susceptible wildlife and/or commercial domestic pig population.

Figure 4 shows the hypothetical pathways of *B. suis* spreading, involving presence or absence of infected WL.

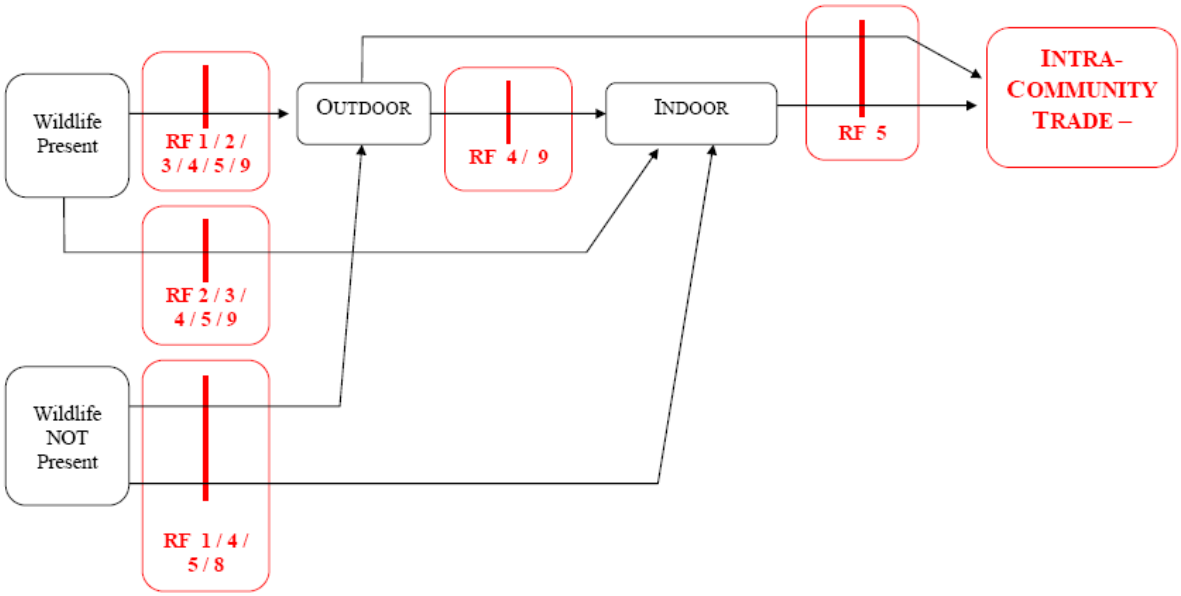

Figure 4. Hypothetical pathways of *B. suis* spreading (see Table 4 for explanations).

Note: whenever RF 2, 5 and 9 are mentioned in the figure, they are considered as being together with RF 6, 7 and 10, respectively

Figure 4 graphically shows potential risk factors that may play a role in the local introduction and further dissemination of the infection. However, the extent to which each of these factors may contribute to these events would vary on a factor by factor basis. In turn, this would have an effect on the likelihood of further dissemination. For example, management factor may reduce the likelihood of infection of a pig holding exposed to *B. suis*-infected WL. Other risk

mitigating factors may include control regimes and testing strategies as a risk mitigation measure to prevent further spread of infection from infected holdings to other outdoor or indoor pig holdings.

Table 4. Risk factors for *B. suis* spreading as considered in Figure 4

Code	Risk Factor (RF)
RF1	Housing management
RF2	Low level of biosecurity
RF3	Direct or indirect contact with infected wild boar, free-ranging pigs or hares
RF4	Purchasing animals or semen without testing
RF5	No testing of live pigs
RF6	Low level of Good Health Practices (GHP) implementation
RF7	Lack of detection of inapparent infection
RF8	Contamination of semen collection centres and equipment
RF9	Contamination of transport vehicles
RF10	Transport of pigs from different holdings, mixing of pigs

8.3. Description of identified Risk Factors

RF1 - Housing management

The likelihood of the introduction of the infection from potentially infected wild boar, free-ranging pigs or hares and its establishment in outdoor and backyard pig population would depend on:

a) Type of housing management (outdoor vs indoor).

b) Potential for effective direct or indirect contact with infected wild boar or free-ranging pigs.

c) Effective dose to initiate infection (as the exposure to *B. suis* may not necessarily result in initiating infection in all exposed pigs).

B. suis was reported in a limited number of outdoor pig holdings only in the affected MS over a period of time. In most cases, the introduction of the infection to an index holding was attributed to contact with infected wild boar. Further dissemination of the infection to subsequent holdings was attributed to onward transmission from the index holding. The type of potential transmission has not been clarified.

Recent experience in Germany suggests that all affected holdings housed pigs outdoor. Holdings situated in or close to wood or forests would appear to be at higher risk of infection.

There are no recent or current official reports of the infection being detected in pigs housed indoor.

RF2 – Low level of biosecurity

The likelihood of the introduction of the infection directly from potentially infected wild boar, free-ranging pigs or hares and its establishment in pig populations would depend on:

a) Level of biosecurity.

b) Potential for effective direct or indirect contact with infected wild boar or free-ranging pigs.

c) Survival of *B. suis* in the environment.

d) Effective dose to initiate infection (as the exposure to *B. suis* may not necessarily result in initiating infection in all exposed pigs).

The introduction of the infection may happen indirectly via contaminated feeding, grass, straw or litter and the likelihood of indirect/direct transmission would depend on several conditions. These are:

a) Level of biosecurity.

b) Feeding practices (*i.e.* home prepared food vs commercial food).

c) Potential for contamination.

d) Survival of *B. suis* in the environment.

e) Effective dose to initiate infection (as exposure to *B. suis* may not necessarily result in initiating infection in all cases).

Biosecurity level will depend on the type of housing. Low level of biosecurity in outdoor holdings would mean that pigs may be relatively easily exposed to a pathogen due to absence of effective physical separation (*i.e.* fencing). Unchecked food (food contaminated with *B. suis*), unrestricted human movements and unrestricted contact with contaminated environment may occur in outdoor and indoor holdings.

Outdoor domestic pig farming is referred to fenced holdings with appropriate sanitary measures in place and controlled access to the holding but with direct contact to the environment. This way of farming can be found in various MS.

Infection of wild boar with *B. suis* has been recognised in Mecklenburg-West Pomerania (MV), Germany, for decades and there is awareness of *B. suis* within the veterinary services. Seven outbreaks have occurred in domestic pigs since 1991: one in 2004 and 6 in four districts in 2008. All *B. suis* outbreaks in MV happened in organic pig holdings where the pigs were kept outdoors or close to woods throughout the year. The number of organic pig holdings (outdoors) has increased in MV during the last 5 years. In 2008 there were 10 organic pig holdings, each with more than 100 sows, managed as outdoor holdings. All these holdings have to follow very strict hygienic rules: double fencing, change of clothes, storage of straw within the holding etc. However, in 2008, 6 out of these 10 holdings were found infected with *B. suis*.

Epidemiological investigation into the transmission of *B. suis* in this area have excluded contact via people or transport as the cause. In addition, all breeding pigs tested negative for brucellosis before entering the holdings. It has therefore been considered that indirect contact with the infected environment may have resulted in *B. suis* transmission.

In indoor housing, pigs are kept within stables during their whole life. Some farmers may breed and rear their own pigs up to slaughter. Other farmers may purchase young pigs (‘weaners’) from breeder holdings and fatten them (‘finishers’). The majority of commercial pig farmers buy their replacement sows and boars from specialized units, called ‘multipliers’. These holdings produce crossbred animals which produce young piglets which grow very well. There are fewer multiplier holdings than commercial pig holdings. The multipliers purchase their purebred sows and boars from ‘nucleus’ herds. There are even fewer of this type of herds. For this reason the pig industry is said to operate as a ‘pyramid structure’ with large numbers of commercial pig holdings at the base of the pyramid and much smaller numbers of nucleus herds at the top.

RF3 – Direct or indirect contact with infected wild boar, free-ranging pigs or hares

The introduction of the infection from potentially infected wild boar, free-ranging pigs or hares and its establishment in free-ranging and backyard pig population would depend on:

a) Known presence of *B. suis* in wild boar, hares and in free-ranging pigs in certain locations in some MS.

b) Potential for effective contact and transmission (sufficient infection dose) of *B. suis* infection to domestic pigs.

Foci of *B. suis* have been reported in wild boar and hares populations in limited areas in some EU MS. Majority of the available evidence suggesting the potential higher prevalence of infection in these populations is based on serological studies. These data could suggest that exposure to infection may have resulted in the spread of infection within the local population. However, these findings would have to be considered in the context of the reported very small percentage of *B. suis* isolates obtained from wild boar and the known potential for serological tests for *B. suis* to cross-react to some other pathogens (*e.g. Yersinia* spp., *E. coli* spp., *Salmonella* spp.). In any case, some serological studies in France (Appendix 1) over a period of time may suggest that the prevalence of *B. suis* infection appears to be relatively stable in the affected local population of wild boar. This seems to be also the case in Spain, and similar results could be seen in the north-eastern part of Germany. Similar considerations may apply to hares. Limited evidence suggests that these foci may closely be associated with specific ecological conditions that favour establishment and maintenance of infection.

Free-ranging pigs

While *B. suis* infection is known to be present in wild boars and hares, it remains uncertain to what extent it may be present in free-ranging pigs in the EU. Some traditional keeping systems have remained in some MS, for example:

East Balkan pigs in Bulgaria. This ancient breed was established some 2,500 years ago. Farming of these pigs is now restricted to 12 municipalities in 3 Districts of Bulgaria. Keeping these animals on pasture is only allowed during the daylight and if a pig-guard is present full time. These pigs are known to have close contact with wild boars, therefore, it is assumed that some of them may be exposed to *B. suis*.

Iberian pigs in Portugal and Spain. Available evidence suggests that *B. suis* has been reported in the domestic Iberian pigs that are reared extensively in certain areas of Portugal and Spain. The apparent prevalence of infection in these pigs appears to be generally high, probably due to the lack of fencing and the easy contacts with potentially infected wild boars. The seroprevalence reported in wild boar in these affected regions appears to be between 30 to 40%. Therefore, *B. suis* infection in the Iberian pig producing regions of Andalucia, Castile Leon and Extremadura (Muñoz, 2008), may be difficult to control.

In Romania, free-ranging pigs are mainly found in the south eastern and eastern parts of Romania especially in the eastern part of country (Danube delta) where they are kept temporary or permanently outdoors. In Transylvania (western part of the country) outdoor pigs are often found together with sheep herds in the mountainous areas. Pigs reared free in Danube Delta or marshes around the Delta are domestic pigs that can be assimilated with wild boar.

RF4 – Purchasing animals or semen without testing

The likelihood of introduction of *B. suis* infection if live pigs or semen donors originating from the affected areas are introduced to pig holdings and are not tested for *B. suis* would depend on:

a) *B. suis* presence in live pigs and semen donors in the affected area.

b) *B. suis* is present in live pigs and semen donors and remains unnoticed.

c) Movement of infected live pigs and semen donors in the affected area (and beyond) without testing for *B. suis*.

d) Whether any commercial pig operation would use back-yard pig semen donors for insemination purposes (*i.e.* natural or artificial).

Purchasing of potentially infected live non-commercial (*i.e.* backyard) domestic pigs originating from infected areas may play an important role in the introduction and dissemination of the infection locally, rather than at the EU level. Nevertheless, movement of these live non-commercial pigs outside the affected area and to other MS cannot be excluded.

Movement of potentially infected domestic boar originating from infected areas for natural breeding may play an important role in introduction and dissemination of the infection locally, rather than at the EU level.

We have no information to which extent any commercial pig operation in the EU would use untested pig semen donors in their operations.

The intensive intra-Community trade with breeding pigs and semen is the background on which the potential risk of spread of *B. suis* should be considered. In 2004-2008, a total of 77,723 consigments of breeding pigs and 24,831 consignments of semen have been registered into the TRACES data base (data received on February 2009 by courtesy of the EU Commission, DG Health and Consumer Protection, Unit D1 – Animal Health and Standing Committees, TRACES Sector). The intensity, connectedness and directions of trade among the countries with the two types of commodities is visualised by directed graphs (Figure 5). The graphs show also the changes in numbers of consignments when comparing the first two amd last two reporting years.

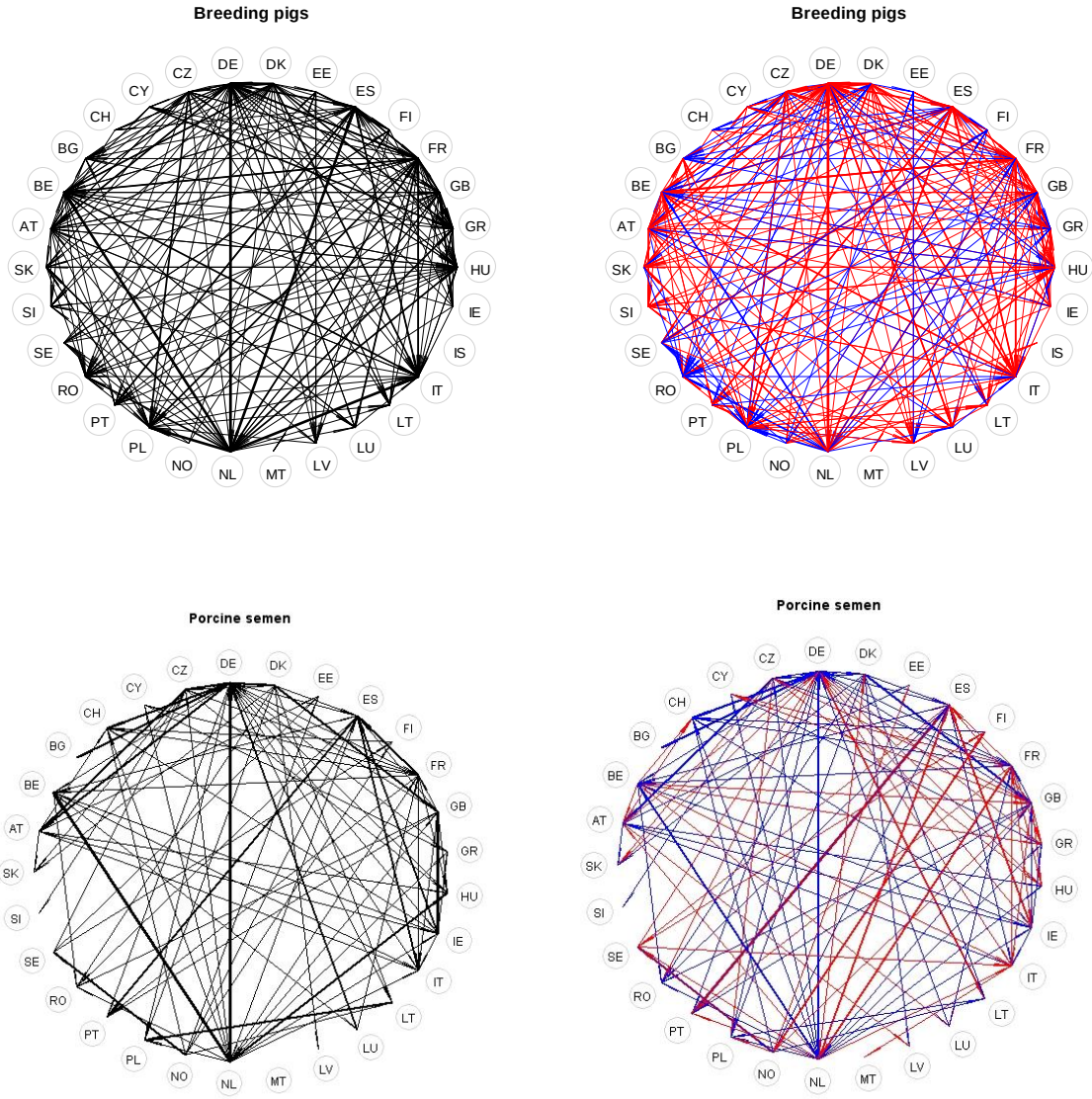

Figure 5. Graphical representation of the intra-Community trade with breeding pigs (top graphs) and semen (bottom graphs) according to TRACES data for 2004-2008.

Left graphs show directed connections (arrows from source to destination) with widths proportional to log10 of the number of consignements. Right graphs show increase (blue) or decrease (red) number of consignments when comparing the periods 2004-2006 and 2007-2008.

RF5 – No testing of live pigs

Given its nature (potential for spreading the infection from infected areas to areas that are currently not infected), this RF is addressed under RF4.

RF6 – Husbandry systems

Given its nature (low level of Good Health Practices (GHP) implementation, Lack of herd health program to deal with diseases in the herd), this RF is addressed under RF1.

RF7 – Lack of detection of inapparent infection

Given its nature (lack of regular testing or missing the infected animals due to imperfection in the testing procedure), this RF is addressed under RF4.

RF8 – Contamination of semen collection centres and equipment

Semen collection centres

Since there is the potential that semen collection centres premises and equipment to become contaminated with *B. suis*, EU rules require such centres to be subject to high level of biosecurity, including cleaning and disinfection and regular testing. If a biosecurity failure occurs (including testing failure) resulting in the introduction and establishment of *B. suis* in the centre, the likelihood of infection dissemination would depend on the following conditions:

a) If infected semen donors get introduced into a semen collection centre.

b) If contamination occurs and appropriate cleaning and disinfection procedures are not followed.

c) If tests fail to detect infection prior to introduction.

d) If semen from such donors is collected and distributed to commercial pig holdings.

RF9 – Contamination of transport vehicles

Broadly, this likelihood would depend on the following conditions. These are:

a) That transport vehicles were on the affected holding and came into contact with contaminated environment.

b) Within-holding prevalence and concentration of *B. suis* in the environment.

c) Cleaning and disinfection practices.

d) Distance travelled and whether the vehicle was in contact with another pig holding.

e) Survivability of *B. suis* during transport.

f) Effective dose to initiate infection (as exposure to *B. suis* may not necessarily result in initiating infection in all cases).

RF10 – Transport of pigs from different holdings, mixing of pigs

Given its nature, this RF is considered similarly to RF9 and this likelihood would depend on the following:

a) That at least one holding is affected

b) Within-holding prevalence

c) Trading practices from the affected holding

8.4. Approach for qualitative assessment of risk factors

For the qualitative assessment of the level of occurrence and levels of adverse effect, the following terms will apply (modified after OIE, 2008b; EFSA, 2006):

Qualitative levels for the occurrence of a risk factor (RF) (note: this is not related to the adverse effect of the factor)	
Negligible	No evidence exists for occurrence OR some evidence for occurrence on levels <0.1% of units* exists
Low	No evidence for occurrence yet but infrequent occurrence should be expected OR some evidence for occurrence on levels between 0.1% and <5% of the units of the factor
Medium	Some evidence for occurrence on levels between 5% and <10% of units of the factor
High	Some evidence for occurrence on levels of ≥ 10% of units of the factor

Qualitative levels for the adverse effect of a risk factor (RF) in terms of likelihood (L) of B. suis infection becoming established in a domestic pig holding given that the holding is exposed to the RF	
Negligible	The likelihood of B. suis to be established given exposure to the RF is not changed in any perceivable way
Low	The likelihood of B. suis to be established given exposure to the RF is at a slightly increased level on a population average; *B. suis* infection could become established in a small number of holdings exposed to the RF
Medium	The likelihood of B. suis to be established given exposure to the RF is at increased level on a population average; *B. suis* infection could become established in about half of the holdings exposed to the RF
High	The likelihood of B. suis to be established given exposure to the RF is significantly increased on a population average; *B. suis* infection could become established in more than half of the holdings exposed to the RF

* Units are herds or pig holdings in general. For some risk factors, different definitions would apply (see Chapter 8.5 for details).

On the basis of available information presented in other sections above, we have identified a number of potential RF and assessed them in a qualitative manner. This assessment considers a likelihood for occurrence for each RF and its potential for impact. This assessment also takes into account uncertainties and assumed variability (heterogeneity) within pig population in MS and a range of assumed locally prevailing conditions. This approach was taken because information on most RF remains uncertain/or incomplete. The evidence to support assessment of each RF is outlined below and is based on available reports from some MS.

Overall summary scores for each RF are established under the assumption that (according to instructions given) the ranges (min-max scores) given by each individual expert reflect variability within or among MS and that differences among experts reflect uncertainty. However, it was taken into account that ranges for some RF given by experts may also reflect uncertainty. Some members of the WG evaluated the RF by scores.

The qualitative scores for occurrence (Occ) and adverse effect (Eff) for each RF and each expert is summarized using a 4x4 matrix where rows and columns represent the four levels for adverse effect and occurrence, respectively. We refer to this matrix as a "response surface". The scoring system also allows experts to express that there is very little variability, in which case the minimum level and maximum level are identical. For example, expert A wishes to express that the level of occurrence for one RF is always Low and that the level of the adverse effects is min=Low and max=Medium, then the cell of the response surface corresponding to this combination (Occ=low, Eff=low, medium) assumes the value "0.5" and all other cells have the value "0". Assume expert B scores occurrence as min=Negligible and max=Low. For

adverse effect, she scores min=Negligible and max=High. In this case, all cells are zero except the cells corresponding to Occurrence < Medium with cell values of 0.125. The cell total of the scoring from one expert is 1.0. The summary response surface of experts A and B combined indicates that the Occurrence=Low and Effect=Low, Medium has the highest support. The spread of cell values in the combined table for expert A and B indicates the uncertainty arising from eliciting the information from the two experts (see

Figure 6).

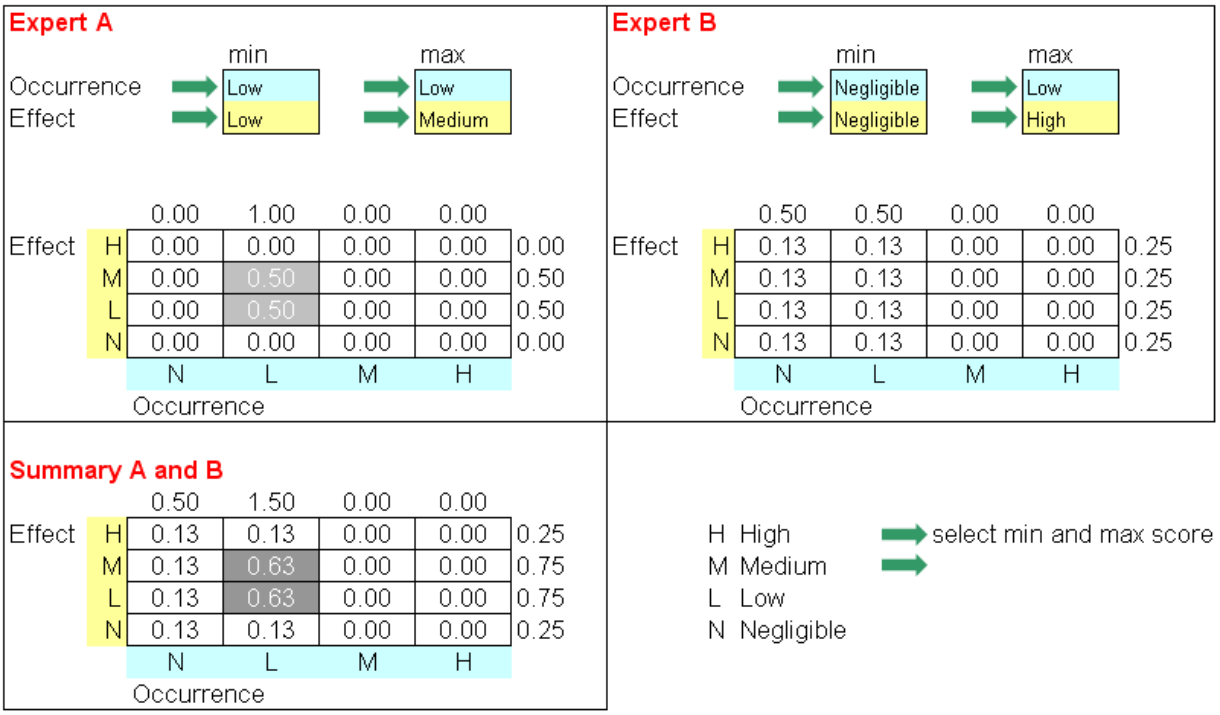

Figure 6. Illustration of the qualitative scoring system for levels of occurrence and adverse effect of a risk factor.

(N=Negligible, L=Low, M=Medium and H=High)

The marginal totals are derived from the ranges indicated by experts (e.g., expert A indicated min=max=Low for occurrence and expert B scored min=Negligible and max=High for occurrence). The cell values of the 4x4 matrix are referred to as "response surface". See text for detailed description.

Mathematically, the cell values X_{ij} are defined as $X_i * X_j$, where i,j=(1,2,3,4) are indices denoting the row and column number and X_i and X_j denote the marginal totals for rows and columns, respectively. The four values of the marginal totals X_i and X_j are defined such that they sum to unity and are zero if the corresponding score level is not included in the score range (min, max, given by expert) and uniformly distributed and nonzero if the corresponding score level is included in the score range. Summary scores for multiple experts are obtained by summing up the X_{ij} cell values of each individual expert. The overall total for the summary response surface equals the number of experts that contributed to the assessment.

For the purpose of ranking RF, the summary response surfaces are totalled whereby each cell ij receives a weight of $w_i * w_j$. These weights are 1, 2, 3 and 4 for the levels Negligible, Low, Medium and High, respectively. This summary score should not be interpreted for any other purpose than for ranking.

8.5. Results of the assessment of risk factors

The summary response surfaces for the ten identified risk factors are shown in Figure 7. The grey scale and the numbers printed in the shaded cells corresponds to the support given by qualiltative expert judgement for thre respective combination of level of occurrence (horizontal scale) and level of adverse effect (vertical scale). The purpose of this representation is to maintain different opinions of the experts, which could reflect differences in the locally or regionally prevailaing situations. The assessment in its totality addresses the situation in the whole EU.

- RF1 (Housing management) was assessed with negligible or low level of occurrence and low to high level of adverse effect.
- RF2 (Low level of biosecurity) and RF3 (Direct or indirect contact with infected wild boar, free-ranging pigs or hares) were assessed with a wide range (negligible through high) level of occurrence and medium to high level of adverse effect. There was indication of a heterogenous assessment by experts with also a considerable support for the combination of high level of occurrence and high level of adverse effect. It is assumed that this is an expression for the view that in some areas or production systems the lack of biosecurity and direct contact with infected wild life species may occur frequently.
- RF4 (Purchasing animals or semen without testing) was assessed with negligible or low level of occurrence and medium to high level of adverse effect.
- RF5 (No testing of live pigs done) was assessed with low to high level of occurrence and low to high level of adverse effect.
- RF6 (Low level of Good Health Practices (GHP) implementation) was assessed with low to high level of occurrence and medium to high level of adverse effect.
- RF7 (Lack of detection of inapparent infection) was assessed with low to high level of occurrence and medium to high level of adverse effect.
- RF8 (Contamination of semen collection centres and equipment) was assessed with negligible to medium level of occurrence and low to high level of adverse effect.
- RF9 (Contamination of transport vehicles) was assessed with negligible or low level of occurrence and negligible or low level of adverse effect.
- RF10 (Transport of pigs from different holdings, mixing of pigs) was assessed with negligible to high level of occurrence and low to high level of adverse effect.
- According to a summary score, RF7 seems to be the most important one and RF9 the least important.

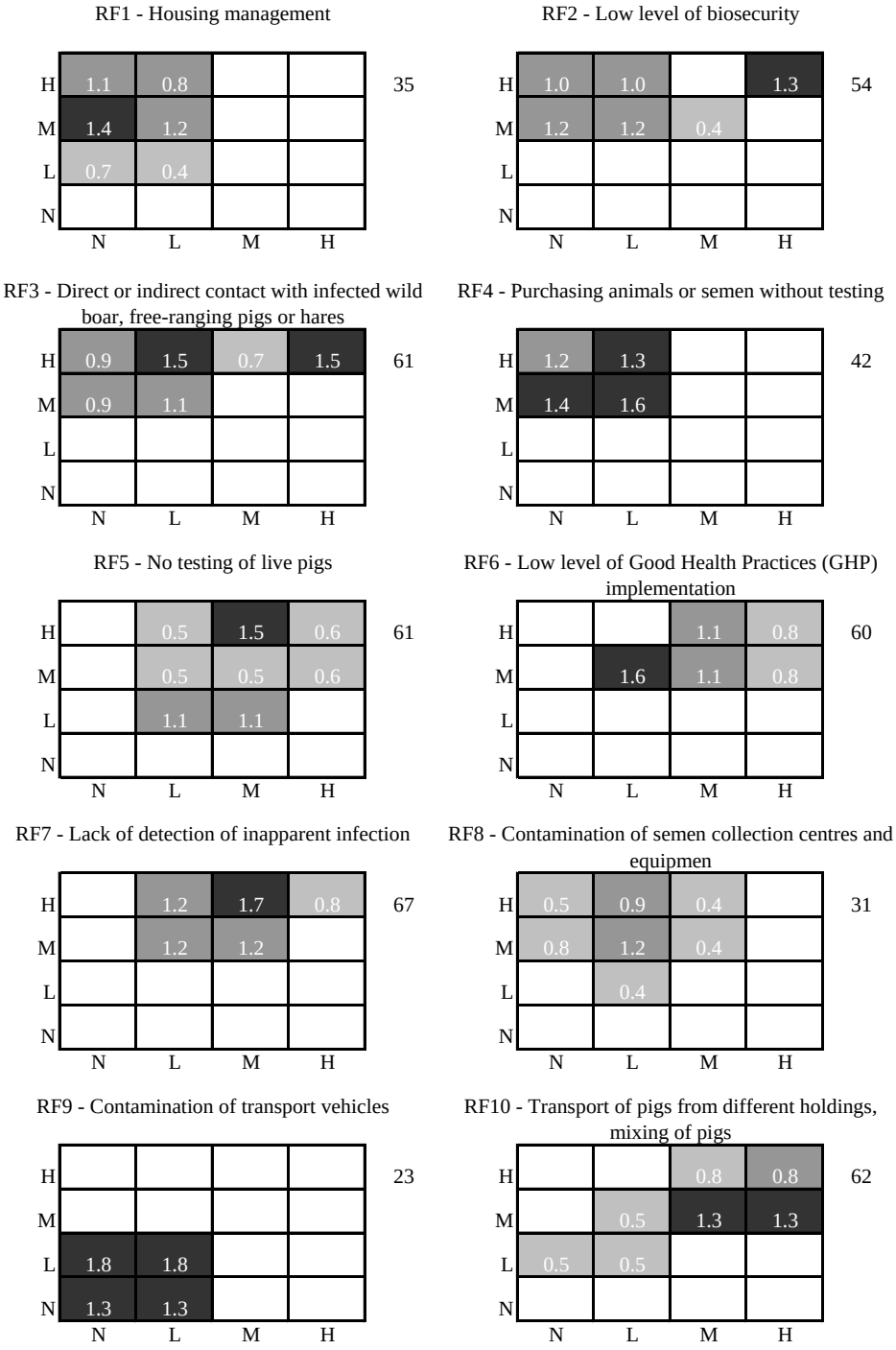

Figure 7. Summary of scoring on qualitative levels of occurrence (x-scale) and adverse effect (y-scale) for ten risk factors (RF) elicited from seven experts of the working group.

RF- see description in the text

The qualitative levels are Negligible (N), Low (L), Medium (M) and High (H). The cell values are visualised using a three-level gray scale and only shown for values grater than 0.4. The cell values reflect how many experts would have chosen the respective combination of qualitative occurrence and effect level with their indicated range of variability. Right to each response table, a summary score is given for the purpose of ranking, which is the total of each cells weighted with the values w=1,2,3,4 for the respective qualitative score levels.

Conclusions

Currently affected areas

Based on limited information, *B. suis* infection seems to be only confined to wild boar and hares in certain limited areas of some MS. This is based on limited information available mainly from serological testing carried out in certain MS.

Following recent sporadic introduction to local domestic pigs housed outdoor in Germany, there is no further evidence to suggest that *B. suis* was distributed over a wider area. There is also no evidence to suggest that the currently affected areas in the EU are expanding.

The presence of infected boar (and hares) and potential for exposure of outdoor pig holdings remain the most important risk factors in the currently affected areas. Exposure to infected wild boar would be influenced by the existing level of biosecurity resulting in various levels of potential for either direct or indirect contact. In contrast to high level of biosecurity, low level of biosecurity would increase potential for the contact. In addition to the level of biosecurity, direct contact would also be influenced by the type of pig housing (*e.g.* outdoor vs indoor). Recent cases of *B. suis* infection in Germany were detected only in pigs housed outdoor, close to areas with wild boar.

Should *B. suis* infection be introduced to holdings which do not join the intra-Community trade (*e.g.* backyard), the most important risk factor would be potential for failing to recognise and report the presence of the infection. This could create the potential for further dissemination as such introduction would result in spread within the herd or to other similar herds. Usually, pigs from such holdings do not enter the industrial pig production system, but in local and special situations this may occur. The most important direct contact would be by exchange of infected pigs or use of infected boar for insemination. Indirect contact would mainly depend on mechanical transmission by people and shared contaminated equipment. The role of other potential for mechanical transmission (*e.g.* rodents, scavenging birds) remains hypothetical.

Potential for wider spread in the EU

With the exception of specific cases of *B. suis* recently reported from Romania, there is no evidence of any increased potential for the *B. suis* infection spread to unaffected areas in the EU. It still remains unclear how this introduction may have occurred.

Any potential for the introduction of *B. suis* infection to outdoor pigs would be primarily influenced by the level of biosecurity and the testing practices in place. Should the infection become established in holdings participating the intra-Community trade (*e.g.* outdoor, indoor, semen collection centres), the most important risk factor for wider spread within the EU would be if the infection remains unrecognised. This would create the potential for further spread within the EU either by direct or indirect contact. In case of direct contact, movements of live pigs (mainly breeding pigs) and semen would be the most important risk factor given the intensive level of intra-Community trade.

Recommendation

Given the important role of the wild boars as potential source of *B. suis* infection for pigs kept in outdoor holdings with insufficient biosecurity, further risk assessment will require more complete and reliable data on the infection prevalence in wild boars collected using standardised serological tests, bacteriological culture and typing of isolates.

9. Strategies for the control of porcine brucellosis

This section will consider the control strategies applicable to the general pig population (Chapter 9.1) in the EU and then will focus on the particular population of boars admitted (Chapter 9.2) and kept (Chapter 9.3) in Semen Collection Centres (SCC).

9.1. General control strategies

There are several control measures that may be applied to reduce the spread of the *B. suis* infection within and among pig holdings. Most of these measures individually cannot accomplish effective control level, thus combining these measures could improve the control system. Some of the most important approaches for pig brucellosis control in attempting eradication may be through test and slaughter, vaccination, or a combination of both.

Eradication requires the identification of infected animals, their progressive elimination from the herd and replacement with non-infected animals (Alton, 1990; Crespo Léon and Ferri, 2004) and avoiding contacts to infected herds or wild boars/hares. Free herds may be protected from disease introduction by testing all animals before introduction. In countries where herds have been freed from brucellosis, another approach is used: infected herds are identified and slaughtering the whole herd is conducted, followed by re-population with non-infected animals (Alton, 1990).

In the absence of eradication procedures other than depopulation, control of the disease would be more readily achieved if effective vaccines were available (Alton, 1990). Characteristics and limitations of currently available vaccines for porcine brucellosis (*B. suis*) are discussed in Chapter 4. ("Pathogenesis of *B. suis* infection").

Conclusions

There is a lack of epidemiologic data on porcine brucellosis in most MS because the occurrence of the disease is mainly sporadic (with the exception of certain areas where the characteristics of the production systems allow *B. suis* to be endemic). It should be emphasised that there is no standardised testing protocol for *Brucella* infection among MS. Moreover, considerable methodological variation exists regarding the detection and confirmation of *B. suis*. Thus, there is a potential of under-reporting and lack of awareness of this infection.

Within the EU, the epidemiological situation is varied, with countries free from the disease, some reporting sporadic outbreaks and others reporting this disease as an emergent problem.

Available epidemiological evidence shows that *B. suis* biovar 2 is the most common agent, while biovars 1 and 3 can also occur.

B. suis biovar 2 has also been reported in *Sus scrofa* (wild boar) populations in certain areas in some MS. *B. suis* biovar 2 might also be harboured by hare (*Lepus* spp.).

For some MS data for the incidence rate and space distribution of *B. suis* in wild boars and hares are available, but similar data is lacking for most EU MS and therefore the epidemiological situation is not clear.

There is little knowledge on the persistence of *B. suis* outside the host.

Historically hares in some MS were reported as the source of repeated outbreaks in pigs, mainly as a result of swill feeding offal from hunted hares. The education of hunters and

intensive efforts to prevent swill feeding is considered a major reason to the decrease in outbreaks in swine originating from hares. The prevalence of the infection in hares can be a link also to the infection of wild boars. Available evidence suggests that currently the wild boar seems to remain the main source of infection for domestic pigs because several outbreaks of *B. suis* occurred in outdoor rearing systems, even on fenced premises, with the source of infection traced to contacts with wild boars. This outdoor housing is used in "open air" commercial systems or in extensive systems (as the free ranging pigs Iberian pig rearing system). Transmission from wild boars to pigs is suspected to be through the venereal route, as crossed piglets (striped) have been reported at least in France and Portugal. Other routes might as well be possible.

Hares have been considered as a possible source of *B. suis* outbreaks in domestic pigs by the oral transmission route via swill feeding including offal from hunted infected wild boars or hares.

Some reported outbreaks have also been traced to the introduction of infected live animals originating from holdings where the diseases had not been detected.

Semen production seems to be well controlled and follows legislation requirements for the introduction of boars in SCCs, continuous surveillance of the disease and semen preparation requirements. However, transmission through this route could constitute an important way of disease dissemination (see Sections 9.2 and 9.3).

Recommendations

Outbreak investigation and the isolation of the strain involved in *B. suis* outbreaks in domestic pigs and in wildlife should be attempted, whenever possible, in MS, for acquiring a more consistent epidemiological profile of the disease. Outbreak investigations should include wild boar and/or hare populations if present in proximity of the index herd.

Controlling this disease may require intensive efforts to reduce its spread from wild animal species. The control strategy, therefore, should consider its reduction or (where possible) eradication of the disease from wild life species.

In order to form a clearer picture of the epidemiological situation, the role of wildlife should be investigated. It is important to estimate the prevalence of *B. suis*, to obtain more reliable information about distribution of the infection and to identify the biovar(s) circulating.

Studies on the dynamic of wild boar population should be encouraged as they may provide baseline information not only for *B. suis* epidemiology, but also for other diseases as Classical Swine Fever.

Appropriate surveillance methods should be used to substantiate freedom or low prevalence epidemiological status in wild boar populations (*e.g.*, demonstrating that prevalence is below 5% with 95% confidence).

The concerned stakeholders should be made aware that the *B. suis* infection risk is an additional consideration for banning swill feeding including offals from hunted and potentially infected wild boar and hares.

According to the level of infection in wildlife (wild boars and hares) in terms of prevalence and space distribution, the following strategies for outdoor farming could be considered, under consideration of local epidemiological circumstances:

1. In MS (or regions of MS) where wild boars and/or hares are free from *B. suis* infection, outdoor farming could continue under the existing conditions.
2. In MS (or regions of MS) where the presence of *B. suis* infection in wildlife has been confirmed, outdoor keeping could be considered when certain additional measures are fulfilled: improvement of bio-security measures, awareness of the herd owners (especially as far as the early detection of abortion is concerned), introduction of a testing regime and when possible a canalization of trade (*i.e.* pigs admitted only directly to the slaughterhouse). A clear indication of non-effective fencing is for example the occurrence of domestic-wild boar crossbreds in or around an outdoor holding. Outdoor keeping could be considered only in holdings without in-house breeding activity (*i.e.* no sows and boars are present in this outdoor type of holdings). Furthermore, movement of animals from outdoor systems to indoor systems should be avoided. Local competent authorities may consider movement restrictions from holdings with higher risk status to holdings with lower risk status.

In domestic pig holdings or epidemiological units where *B. suis* has been isolated or confirmed, it is recommended to slaughter infected pigs. It should be considered to apply whole-herd slaughter as this would reduce the risk of circulation within and spread to other holdings of *B. suis* due to undetected infection. According to the EU Regulation 854/2004, these animals are to be slaughtered separately from other animals and the udder, genital tract and blood must be declared unfit for human consumption, where the meat coming from animals in which post-mortem inspection has revealed lesions indicating acute infection with brucellosis is to be declared unfit for human consumption.

Boars kept in semen collection centres should continue to be selected and introduced from holdings that are epidemiologically proven free from *B. suis*. Donors should continue to be serologically tested on holding of origin and in quarantine before being placed in the centre and then on a regular basis (see also recommendations from following sections).

9.2. Diagnostic testing for admission of animals to semen collection centres

Based on the qualitative assessment of risk factors (Figure 7), the lack of testing of boars for admission to and during stayin semen collection centres (RF4) was identified as occurring with negligible or low probability but as having a medium or high adverse effect in terms of transmission. The probability of transmission of *B. suis* by semen can be mitigated by several measures. An explicit testing scenario is described in Annex B to Council Directive 90/429/EEC (see Figure 8). We refer to the testing scheme and decisions taken based on test outcomes as a "protocol for admission" of boars to approved semen collection centres whereby the source holdings are located in the same or in other MS. Boars may also be imported from third countries but this scenario was considered outside the scope of the mandate. The routine compulsory testing of boars during their stay or before exit from the semen collection centre is addressed in the following section. The goal of this section is to describe the utility of the currently approved diagnostic tests and other candidate tests (*i.e.* the current admission protocol and alternative protocols) – in terms of the probability that at least one *B. suis* infected boar of a group of animals from one *B. suis* affected source holding is admitted to a semen collection centre as a result of false negative diagnostic test results obtained during the admission process. We refer to this probability as introduction probability (*PrIntro*) and regard it as one element of the risk to spread *B. suis* by semen. It is noted that this probability is inversely correlated with the probability of rejection of boars sourced from

non-infected source holdings as a result of false positive test results (results not shown). The rejection probability and its associated adverse economic consequences were considered outside the scope of the mandate. The evaluation of the probability *PrIntro* is based on the estimates of the diagnostic sensitivity (Se) and specificity (Sp) described in Chapter 7.

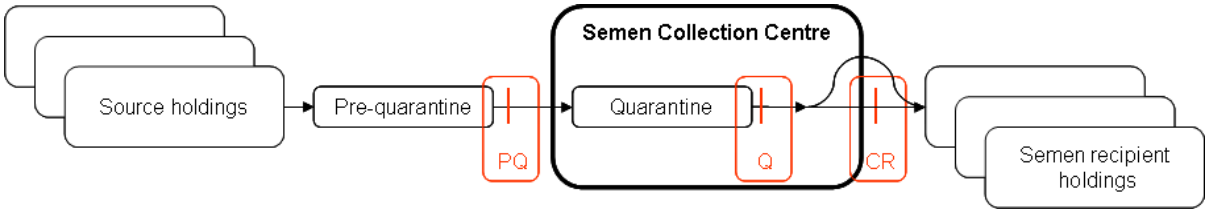

Figure 8. Schematic concept for the admission protocol for boars to a semen collection centre

Admission protocol involving pre-quarantine (PQ), testing in quarantine (Q) and compulsory routine testing (CR). Positive test reactions in PQ and/or Q trigger epidemiological investigations to rule out Brucellosis in source holdings

Boars are admitted to enter a quarantine accommodation of a semen collection centre subject to negative results obtained with the Buffered Plate Agglutination Test (BPAT) for all individual boars within a 30-day period prior to the beginning of the quarantine. In case of positive test results, the reactor animals are not admitted to enter the quarantine whereas animals with negative test results from the same holding are admitted in the quarantine accommodation after the confirmation of the brucellosis free status of the herds or holdings of origin of the positive reactors. This testing is referred to as *pre-quarantine testing* (*PQ*). The two possible outcomes of interest are positive (*PQ*+) if one or more animals react positive with BPAT, which would trigger epidemiological investigations of the source holding and negative (*PQ*–) if all test BPAT results are negative. We presuppose that the epidemiological investigations triggered by BPAT reactions are reliable in confirming or ruling out *B. suis* infection in the source holding. Therefore, the SAT testing result will not contribute to the sensitivity of the admission testing protocol. As one alternative admission protocol, we shall investigate the same scenario with the modification that epidemiological follow-up is only triggered based on positive confirmation by SAT. Furthermore, all boars admitted to the semen collection centre must be tested during the last 15 days of the period of quarantine of at least 30 days with negative results by BPAT. All reactor animals must be tested by a SAT and CFT. On the positive animals, a second series of tests (BPAT, SAT, CFT) is carried out on samples collected more than seven days after the first collection. When the suspicion of brucellosis is ruled out by epidemiological investigation of the holding of origin, the animals negative to the first brucellosis test can be introduced into the centre. Animals positive to one test may be accepted if they react negatively to two series of tests (BPAT, SAT, CFT) carried out with an interval of at least seven days. We summarise these testing activities and decision rules to as *quarantine testing* (*Q*). The two possible outcomes of interest are positive (*Q*+) if one or more animals react positive in both BPAT and SAT, which would trigger epidemiological investigations of the source holding and negative (*Q*–) if either all test results are negative or none of the positive BPAT reactors are confirmed by SAT. For the purpose of our evaluations, we make the assumption that the legally required epidemiological investigations are reliable and effective to rule out *B. suis* infection in the source holding and that the probability of infection of boars during transport, *e.g.*, due to mixing from several source holdings, is negligible. Therefore, the outcomes of the second series of tests triggered by positive confirmation of BPAT reactors using SAT does not contribute to the outcome

probabilities *PrIntro*. Furthermore, in the absence of suitable data to prove otherwise, we presuppose conditional independence of diagnostic tests applied in the scheme. When two tests are used sequentially, *i.e.* the second test is only conducted if the first test was positive, a positive sensitivity covariance will increase the overall Se of the combined test while a positive specificity covariance will decrease the overall Sp of the combined test (Gardner *et al.*, 2000). Thus, our results of overall Se of sequentially applied tests will likely be underestimated while our results for overall Sp will likely be overestimated. Alternative testing strategies, which are currently not described in Annex B to Council Directive 90/429/EEC are evaluated for comparison (Table 5).

Table 5. Current protocol as described in Annex B to Council Directive 90/429/EEC and alternative protocols for admission of boars to semen collection centres depending on outcomes of testing during pre-quarantine (PQ) and quarantine (Q)

Testing[1]	Current protocol	Alternative protocol 1	Alternative protocol 2	Alternative protocol 3	Alternative protocol 4
PQ	Sequential: (BPAT, SAT)	Sequential: BPAT, SAT	Single test: iELISA	Single test: cELISA	Single test: RBT
Q	Sequential: (BPAT, SAT)	Sequential: BPAT, SAT	Single test: iELISA	Single test: cELISA	Single test: RBT

1) PQ testing to be done within 30-day period prior to the beginning of quarantine, Q testing to be done during the last 15 days of the period of quarantine of at least 30 days.
2) BPAT= Buffered Plate Agglutination Test, SAT= serum agglutination test, iELISA= indirect Enzyme-Linked Immunosorbent Assay, cELISA= competitive Enzyme-Linked Immunosorbent Assay, RBT= Rose Bengal test.
3) For sequerntial tests, the epidemiolgocal-follow-up investigations are triggered by BPAT reactors (current protocol) or only by SAT confirmations of BPAT reactors.

The introduction probability *PrIntro* is equivalent to the probability of passing with negative results the pre-quarantine (*PQ–*) and quarantine (*Q–*) testing of one or more *B. suis* infected boars out of a group of *n* animals sourced from an affected holding (H+) (Figure 9). It is noted that this evaluation is conditional on assuming the infected status of the source holding. No assumptions about the prevalence of infected source holdings nor on compliance with the described procedures were made as would be required for a full risk assessment.

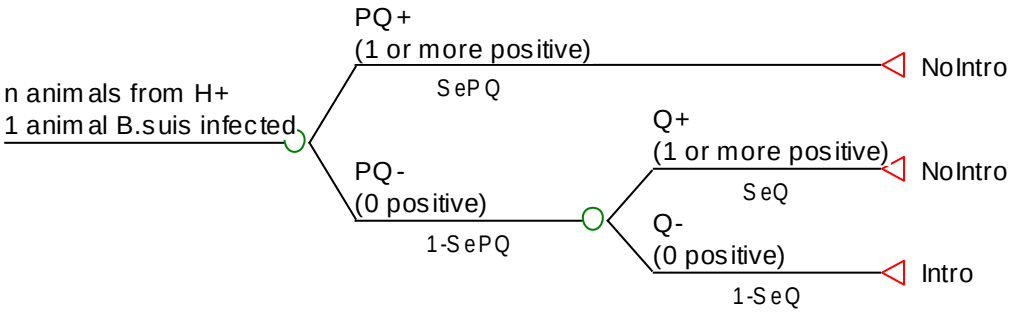

Figure 9. Probability tree for evaluating the probability of introduction (*Intro*) of one or more *B. suis* infected animals from an affected holding (H+) despite diagnostic testing before quarantine (PQ) and during quarantine (Q).

See text for definition of positive (+) and negative (-) outcomes of the testing steps

The introduction probability is evaluated as:

$$PrIntro = (1 - SePQ)\,(1 - SeQ)$$

where SePQ and SeQ are the group-level sensitivities, *i.e.* the probabilities of the outcomes *PQ+* and *Q+* (definitions see above) given that one *B. suis* infected animal is present in the group of n animals sourced from a *Brucella* infected holding. For the current admission protocol a sequential testing scheme is used for both PQ and Q testing whereby BPAT reactors may trigger the epidemiological investigations of the source holding. An alternative interpretation of the sequential testing is that the epidemiological investigations of the source holding are triggered by positive confirmation of BPAT reactors by SAT. The individual animal Se and Sp of the sequential test are then given as SeBPAT*SeSAT and 1–(1–SpBPAT)(1–SpSAT), respectively. These quantities are used to establish the group-level sensitivities SePQ and SeQ, which have identical values due to the same sequential testing scheme used. The calculation of Se and Sp for alternative sequential testing shemes follows the same pattern. For alternative protocols involving only a single test for PQ and Q testing, the respective animal-level Se and Sp values apply. The calculation of SePQ and SeQ uses the exact hyper-geometric distribution model with the modification of assuming exactly one infected animal in the group of n animals sourced from the same holding (Greiner and Paton, 2005). This assumption represents the most conservative assumption about the infection prevalence in the affected source holding. A higher number of infected animals in the group would lead to higher sensitivities and thus lower *PrIntro*. All calculations were done using the posterior distribution of Se and Sp of tests as obtained by systematic review and meta-analysis (Chapter 7). The code for PrIntro is given in Appendix 10.

The results for the current admission protocol show a negative association between group size of the boars sourced from one holding (n) and probability of introduction (*PrIntro*). This can be explained by increase of group-level Se with sample size due to false-positive results (Table 6). This effect is not observed if only tests with perfect Sp are used (results not shown) and less pronounced if tests with almost perfect Sp are used such as iELISA, according to our study results. The 95% uncertainty interval around the estimates of *PrIntro* are based on simulations from the posterior distributions of the Bayesian estimates for Se and Sp of each test decribed in Chapter 7.

Table 6. Estimated probability percent (and 95% simulation interval) of introduction (PrIntro) of one or more *B. suis* infected boars into semen collection centres based on different admission protocols[1]

Group size	Current protocol (%)	Alternative protocol 1(%)	Alternative protocol 2 (%)	Alternative protocol 3 (%)	Alternative protocol 4 (%)
1	6.7 (3.5, 11.6)	27.7 (19.7, 36.8)	0 (0, 0)*	0 (0, 0)*	1.7 (0.6, 3.9)
2	2.7 (0.7, 6.6)	27.5 (19.6, 36.6)	0 (0, 0*	0 (0, 0)*	1.6 (0.6, 3.8)
3	0.5 (0, 3.3)	27.4 (19.5, 36.5)	0 (0, 0) *	0 (0, 0)*	1.6 (0.5, 3.7)
4	0 (0, 1.4)	27.3 (19.4, 36.3)	0 (0, 0) *	0 (0, 0)*	1.5 (0.5, 3.6)
5	0 (0, 0.3)	27.1 (19.3, 36.1)	0 (0, 0) *	0 (0, 0)*	1.4 (0.4, 3.5)

1) See Tab. X1 for description of admission protocols. It is assumed that all boars of the group sourced from one holding and that at least one animal is infected.

2) BPAT= Buffered Plate Agglutination Test, SAT= serum agglutination test, iELISA= indirect Enzyme-Linked Immunosorbent Assay, cELISA= competitive Enzyme-Linked Immunosorbent Assay, RBT= Rose Bengal test.

3) The results of meta-analysis as described in Table 2 and Table 3 were used for simulation of PrIntro.

*The point value and the 95% simulation interval included only the value 0% after rounding.

9.3. Diagnostic testing of animals kept at semen collection centres

The compulsory routine testing of animals in semen collection centres according to Annex B Chapter II to Council Directive 90/429/EEC provides additional safety guarantees for the *Brucella* free status of the centres. Testing has to be carried out either on all animals when leaving the centre, but not later than l2 months after admission where they have not left the centre before this time, or on 25 % of the animals in the centre (samples have to be representative of the total population in the centre) are tested every three months using BPAT. In the latter case, it must be ensured that the samples taken are representative of the total population of the centre and that all animals are tested at least once during their stay at the centre and at least every 12 months if their stay exceeds a year. Positive reactors must be isolated and the semen collected from it since the last negative test may not be the subject of intra-Community trade. Semen collected from each animal at the centre since the date of that animal's last negative test shall be held in separate storage and may not be the subject of intra-Community trade until the health status of the centre has been re-established. We denote the compulsory routine testing as *CR* and differentiate two possible outcomes, *i.e.* positive (*CR*+) if one or more animals react positive with BPAT and negative (*CR*–) if either all test results are negative or, in case of evaluating hypothetical confirmatory tests, none of the positive BPAT reactors is confirmed. The *CR*+ outcome is assumed to trigger follow-up investigations that eventually confirm or rule out *B. suis* infection. We refer this scheme as routine testing protocol. The goal of this section is to describe the utility of the currently approved diagnostic test (BPAT) for the routine testing protocol and other candidate tests in terms of the detection probability (*PrDetect*) to classify the centre as positive given that the within-herd (within-centre) infection prevalence is not less than 5%. We adopted the usual 5% threshold under the assumption that the within-herd infection prevalence would be 5% or higher if *B. suis* becomes established in a SCC. The probability *PrDetect* is equivalent with the so-called confidence and applicable in the absence of any reactors or in the case of re-establishing free status after removal of any reactors. Similar to the rejection probability, the probability of false positive findings, *i.e.* the CR+ outcome in centres that are actually free of porcine Brucellosis could be considered (results not shown). There is a theoretical risk that infectious semen is traded to recipient holdings for example due to false negative confirmatory tests, exit of the reactor animal and continued presence of other inapparently infected boars in the centre, etc. These aspects would require a specific risk assessment, which is beyond the scope of this report.

The detection probability can be defined mathematically as probability to observe the *CR*+ outcome given that 5% of boars or at least one boar in the centre are *B. suis* infected, *i.e.* the centre is positive (C+), which is also the equivalent to the sensitivity of CR testing (SeCR),

$$\text{PrDetect} = \Pr(\text{CR+} \mid \text{C+}) = \text{SeCR}$$

and can be derived as herd-level Se using methods described above.

Table 7. Estimated probability (and 95% simulation interval) of detection of a *B. suis* infected semen collection centre (*PrDetect*) with assumed 5% within-centre prevalence based on different routine testing protocols [1]

Number of boars in centre	Sample size	Current protocol Single test BPAT (%)	Alternative protocol 1 Parallel test: (BPAT, iELISA)(%)	Alternative protocol 2 SIngle test: (iELISA) (%)	Alternative protocol 3 Single test: (cELISA) (%)	Alternative protocol 4 Single test: (RBT) (%)
5	5	100 (94.4, 100)	100 (100, 100)*	100 (100, 100)*	100 (100, 100)*	88 (81.2, 93.3)
10	10	100 (100, 100)*	100 (100, 100)*	100 (100, 100)*	100 (100, 100)*	89.3 (82.4, 94.6)
10	5	65.9 (56.7, 77.7)	73.2 (64.4, 81.3)	50.2 (50.1, 50.2)	55.3 (54.5, 56)	44.6 (41.2, 47.3)
50	50	100 (100, 100) *	100 (100, 100)*	100 (100, 100)*	100 (100, 100)*	
50	25	99 (96.2, 99.9)	99.5 (97.8, 99.9)	82.3 (82.2, 82.3)	91.4 (90.6, 92.3)	79.3 (77.1, 81)

1) See Table 5 for description of routine testing protocols.
2) BPAT = buffered Brucella antigen test, SAT= serum agglutination test,
* The point value and the 95% simulation interval included only the value 100% after rounding.
3) The results of meta-analysis as described in Table 2 and Table 3 were used for simulation of PrDetect.

The results clearly indicate that increasing sample sizes improve the probability of detection (*i.e.* correct positive classification of the centre). However, an increased sample size is also associated with higher rate of false positive outcomes (results not shown). This is a known problem and can only be addressed using tests with better Se and almost perfect Sp, more optimal combination strategies of tests or application of additional highly reliable confirmatory tests. Theoretically, the problem can also be mitigated by choosing an optimal sample size for any given herd size (Greiner and Paton, 2005).

Conclusions

The formal evaluation of the probabilities of correct and incorrect results obtained during the admission protocol or during the compulsory routine testing protocol is hampered by uncertainties related to the limited basis of data for validation of diagnostic tests for detection of *B. suis* infection in pigs.

Little is known about the causes of false positive serological reactions to *B. suis* testing in pigs (FPSR), but it is believed that *Yersinia enterocolitica* O:9 could the main cause of this problem. Having this in consideration, it may be expected that FPSR animals (*i.e.* potentially infected with *Y. enterocolitica* O:9) could be the source of FPSR spread in SCC not yet affected by this phenomenon. On the other hand, the rejection of serological reactors from admission to SCC may have also the consequence to reduce such spread of *Y. enterocolitica* O:9 infection. Such false positive results may potentially have a negative economic impact and other consequences, the assessment of which is, however, beyond the scope of this Opinion.

Very specific tests would be required as confirmatory tests for effective use in the admission protocol and routine testing protocol for SCC. Candidate tests that meet this specification for this purpose may include bacteriological culture of necropsy samples and the brucellin skin test, both interpreted on group-level (*i.e.* group is considered positive if one or more individual positives are found). Due to the absence (for culture) or limitation (for skin test) of validation data for these tests, the probability of introduction of *B. suis* for testing protocols involving these tests could not be evaluated.

It appears that the probability of introduction may be considerable (up to 12%, upper limit of 95% simulation interval) if only a single boar is sourced from an infected holding and

subjected to current testing protocol (BPAT, SAT). The basis for this assessment are the results from the systematic review and meta-anylsis of diagnostic tests for brucellosis in pigs and the assumption that epidemiological investigations triggered by positive reactions of the first test (BPAT) are reliable and effective to rule out *B. suis* infection in the source holding. If such epidemiological investigations were only triggered by positive confirmation of BPAT reactors by SAT, this would result in low sensitivity of the sequential testing protocol and a considerable probability of introduction (more than 36%). The probability of false positive reactors has not formally been addressed.

The iELISA and cELISA appear suitable for use as single tests for testing of boars for admission to semen collection centres, resulting in very low (practically 0%) probability of introduction. These two tests appear also suitable for use as single test for the compulsory routine testing of animals on semen collection centres. However, for the latter purpose, 100% of probability of detection of an affected centre given that 5% infected animals occur can only be achieved with appropriate sample sizes.

The iELISA and cELISA were identified as suitable candidates based on systematic review, meta-analysis and performance evaluation when using this test for admission of boars to semen collection centres and for compulsory rountine testing. However, the test has not been fully evaluated and standardised for use in pigs. The primary reference standards are currently being developed.

Recommendations

After updated review of the diagnostic performance of the fully standardised test, the iELISA and the cELISA may be considered for the purpose of testing boars for admission to semen collection centres and for compulsory routine testing during the stay or on exit from semen collection centres.

To address the FPSR issue it is important to improve the specificity of current diagnostic tests. Specific studies should also be conducted with the aim to identify the mechanisms of transmission of FPSR and to elaborate specific testing protocols to reduce this phenomenon.

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APPENDICES

APPENDIX 1 – DATA ABOUT *B. SUIS* OCCURRENCE IN SOME SELECTED MS

FRANCE

Domestic pig population in France

In 2006, there were 20,990 pig holdings among which 1,681 (8%) were reared outdoor. The structure of pig production in France is given in Table 8.

Table 8. Structure of pig production in France (Source ONCFS/DGAL 2006).

Domestic pig herds	Existing herds	Outdoor pig herds	Breeders Number	Fattening pigs number
Selection	127	7	32 087	147 588
Multiplication	539	37	98 618	446 078
Boar testing stations	3	0	47	41 523
Artificial insemination Centres	33	0	4 204	
Pre AIC Quarantines	37	0	708	
Breeding	2 222	405	195 744	
Breeding-Fattening	7 919	835	899 238	6 023 916
Young breeders collecting centres	19	0	7 598	
Post-weaning	709	27		523 540
Fattening	9 382	370		3 774 618
Total	20 990	1 681	1 238 244	10 957 263

The importance of the "outdoor" pig farming type is given in the following map Figure 10

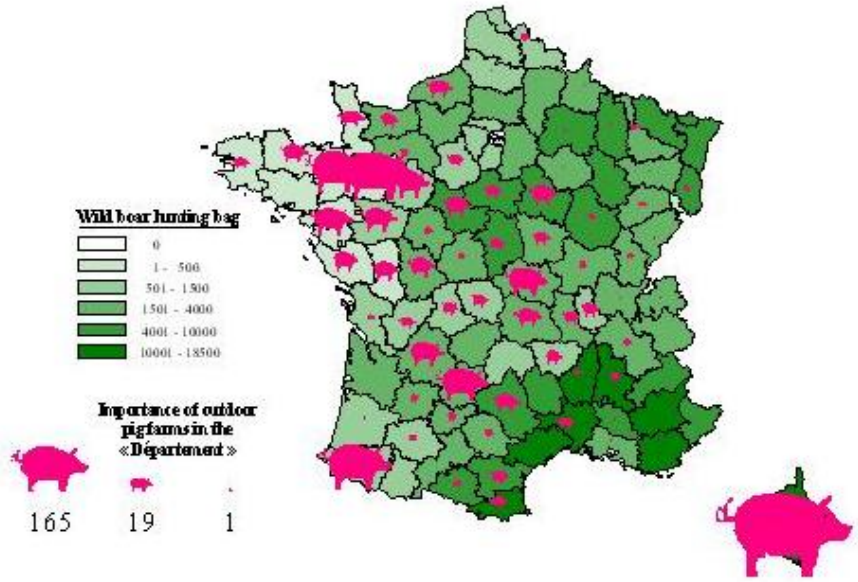

Figure 10. Distribution of outdoor pig holdings in France in comparison with the wild boars hunting bags (2000). Source ONCFS/DGAL.

Brucellosis in domestic pigs in France:

No outbreak of *B. suis* was reported from 1981 to 1993. From 1st January 1993 to 31st December 2008 fifty-nine outbreaks/suspicions reported in mainland France (0-7 confirmed outbreaks/year, Figure 11). Thirty "départements" (French administrative unit – 95 départements in the mainland in total) were concerned (1-4 outbreaks per départements) (Figure 12).

Amongst the 59 outbreaks/suspicions:

- 54 outbreaks confirmed by *Brucella* isolation:
  - 53 due to *B. suis* biovar 2 (98.2%);
  - 1 due to *B. melitensis* biovar 3 (1993)
- 2 outbreaks confirmed by storms of abortions associated with very high within-herd sero-prevalence;
- 3 suspicions based on few seropositive animals and/or few clinical signs (without any other cause identified) with no further confirmation reported.

Amongst the 53 outbreaks due to *B. suis* biovar 2:

- 3 belonged to a cluster (the first outbreak [a research centre] sent infected animals to the 2 other ones [a "testing station" and a Vet. School]) (5.67%).
- All other 50 outbreaks were without any epidemiological link (in particular, no trade of animals, different veterinarians, etc..).

In only one case, hare was considered as the probable source (high density in the area, no wild boar reported in the area).

In all other cases, wild boar was regarded as the probable source. On several occasions crossed piglets and/or observation of wild boar/domestic sow mating were reported. On most occasions, outbreaks were considered to have started more than 6 months before the reporting of the suspicion to the NRL.

Outdoor pig farming in France is similar to industrial farming but without premises (same breeds, mainly Large White and Landrace). Some have lots of sows (sometimes more than 300); AI is generally practiced but usually there are also boars. In outbreaks the fences in place are not proof, neither to wild boars nor hares.

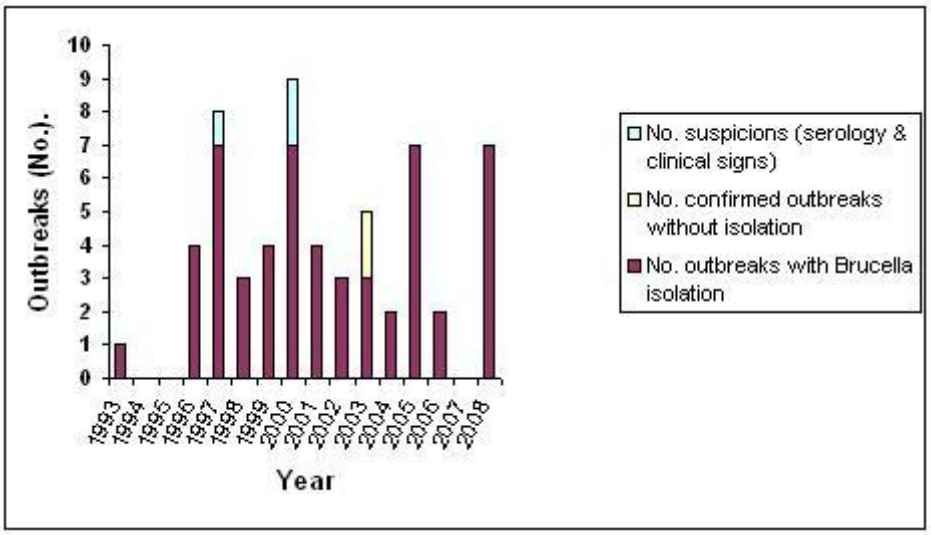

Figure 11. Outbreaks and suspicions of porcine brucellosis in France (1993-2008) (NRL data)

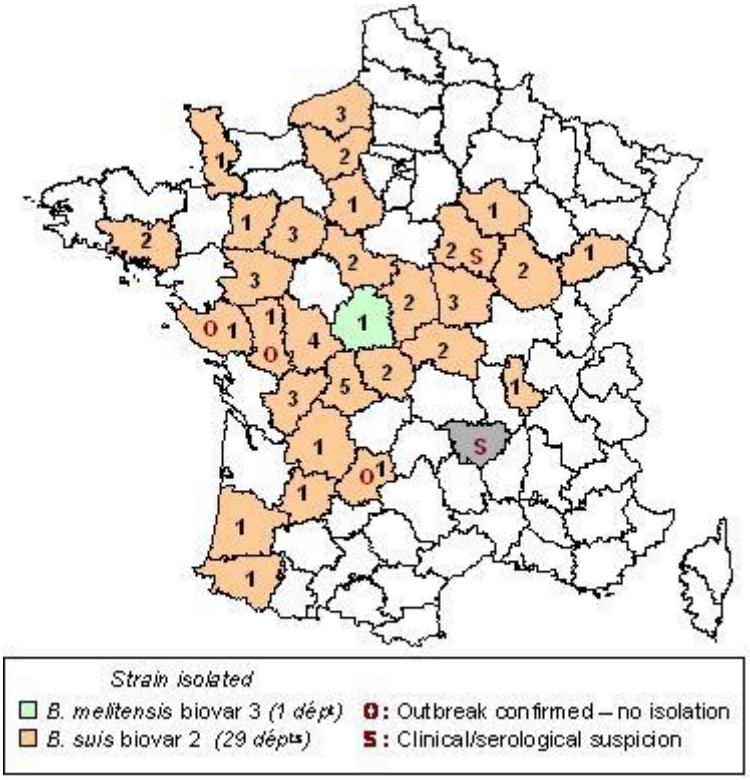

Figure 12. Pig brucellosis outbreaks in France 1993-2008 (NRL data)

Evolution of the Wild Boars population in France.

Ca. 450,000 wild boars have been hunted annually since 2001. It is usually considered that the hunted bag corresponds to 50% of the total wild boar population, *i.e.* the total population is around 900,000-1 million animals. Figure 13 shows the evolution of the national hunted bag from 1973 to 2006. The population has multiplied by ca. 10 in 30 years.

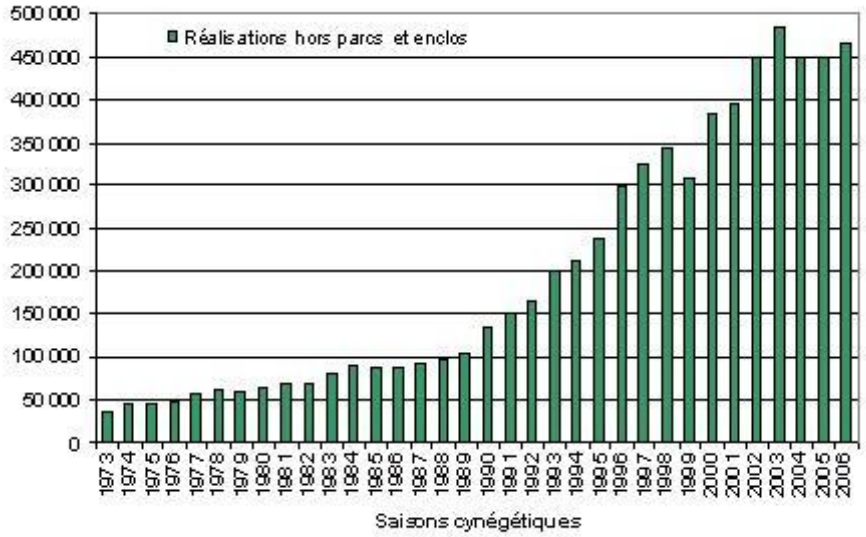

Figure 13. Evolution of the wild boars annual hunting bag (1973-2006) Source ONCFS.

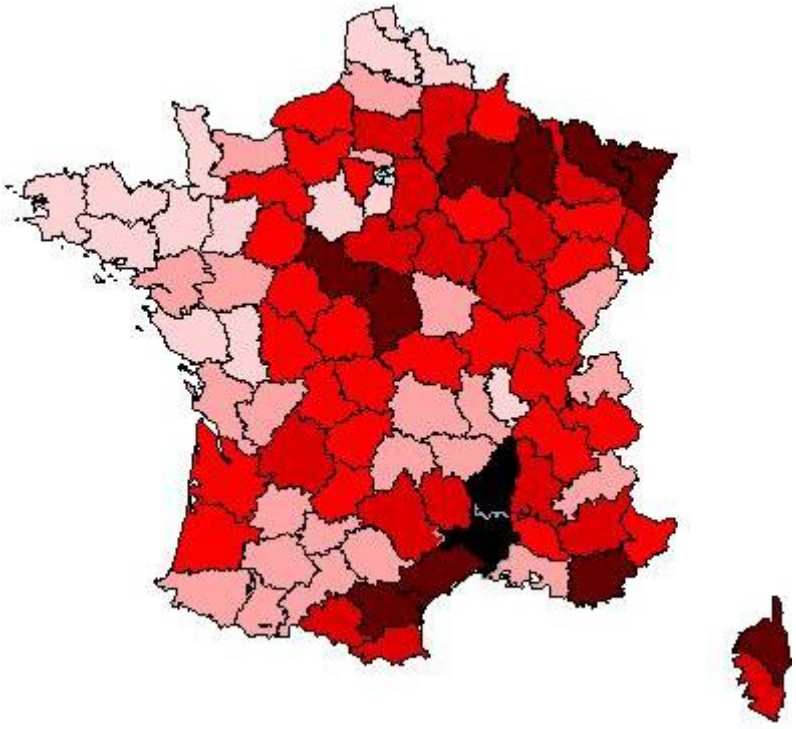

Figure 14. Geographical distribution of the wild boars annual hunting bag (2006) Source ONCFS.

Brucellosis in Wild Boars population in France.

2000-2004 National serological survey (iELISA) (5,842 samples tested) - Mean seroprevalence in >1 year-old wild boars: 48 %.

Table 9. 1996-2001 National serosurveys (RBT and CFT) (France Brucellosis NRL data).

Area (dépts.)	18	56	55	47	31
Years	1996	1997/98	1998/99	1999/00	2000/01
Serological results (n of positive/n of tested)	120 / 344	141 / 487	200 / 624	247 / 797	440 / 1505
Apparent prevalence	36%	29%	32%	31%	29%

Table 10. 1993-2000 National serological/bacteriological surveys (RBT and CFT/*Brucella* culture on spleen) (France Brucellosis NRL data).

Area	Charente (16)	08, 16, 18, 21, 57	Yonne (89)	Tarn (81)	Côte d'Or (21)	Eure (27)	Creuse (23)	Allier (03)	Cher (18)	Meurthe-Moselle (54)
Years	1993	1994	1997-99	1997	1994-97	1997-98	1998	1999	1999	2000
Serological results (n of positive/n of tested)	14 / 32	22 / 61	87 / 233	3 / 34	133 / 430	9 / 37	16 / 54	11 / 52	37 / 99	17 / 67
Apparent prevalence		36%	37%		31%		30%	21%	37%	25%
Bacteriological results (spleen)		7/72 (10%)			3/26 (11%)	6/44 (13%)	4/69 (6%)	10/91 (11%)	5/40 (12%)	4/62 (6%)

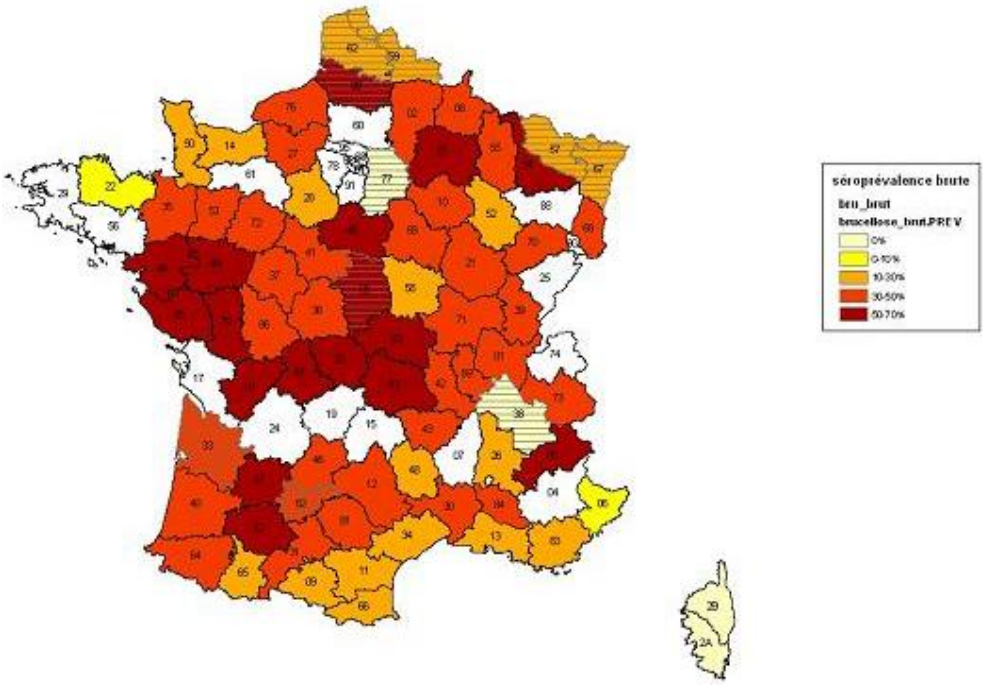

Figure 15. Départemental distribution of brucellosis seroprevalence (adjusted by age and sex).

NB Figure 15: specificity checked in Corsica where brucellosis is assumed to be absent.

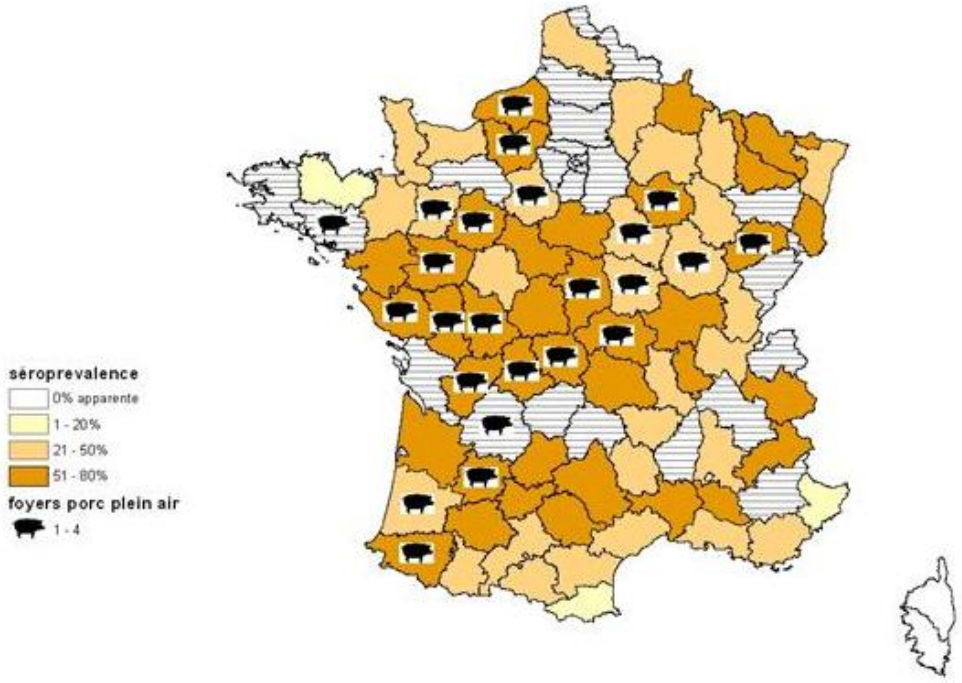

Figure 16. Seroprevalence of brucellosis in wild boars aged more than 1 year and Occurrence of pig brucellosis outbreaks (*B. suis* biovar 2) in outdoor pig holdings in each département (1993-2004).

In Figure 16, the Départements in white did not participate to the survey or did not sample enough animals or did not mention the age of sampled animals

Brucellosis in Hares population in France.

Brucellosis is enzootic in hare populations for years. Several *B. suis* biovar 2 strains are isolated each year. No representative prevalence study performed at national level.

ITALY

After 4 years of wildlife surveillance plan application, the disease surveying activity has improved and spread in the whole Piedmont region. Blood samples and tissue specimens were taken from hunted or dead wild boars (*Sus scrofa*) from Piedmont. These samples were tested by RBT and CFT. Animal tissues were also examined bacteriologically. In 2000-2003, out of a total of 3,406 serum specimens examined by CFT, 234 were found to be positive (6.87%) and 3,172 were negative. From 2,933 serum specimens examined by RBT, 192 were found to be positive (6.55%) and 2,741 were negative. Out of 940 tissue specimens collected for bacteriological isolation, 63 were positive for *B. suis*. In Piedmont, cultural tests confirmed that wild boar brucellosis seropositivity was specific. Based on these data, it was possible to estimate the infection prevalence only in one area, where the disease had been particularly monitored (Gennero *et al.*, 2004).

B. suis biovar 2 was isolated in southern Italy from a male hare (*Lepus europaeus*) imported from Hungary in 1995 (Quaranta *et al.*, 1995).

A study to determine the seroprevalence of brucellosis in hares living in Tuscany provided negative results (Poli *et al.*, 1987). In a study conducted between 1997 and 2000, five hundred sixty-two blood samples were collected from wild boars (*Sus scrofa*) shot in six districts of Tuscany, central Italy. Sera were examined for antibodies specific for *Brucella* spp. by the RBT and iELISA. All the examined sera were negative for anti-*Brucella* antibodies (Ebani *et al.*, 2003).

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SPAIN

The existence of swine brucellosis in Spain is known since 1940, and several outbreaks of disease have been described since then in several Spanish regions. The prevalence of the disease is supposed to be low, but the real situation of the disease in the different pig husbandry systems is largely unknown. The disease is frequently reported in the Iberian pig population, that it is reared in outdoor husbandry systems. The disease is also sporadically

diagnosed in intensive indoor holdings that have epidemiological relationships with outdoor holdings (Leon *et al.*, 1976; Leon *et al.*, 1978; Muñoz *et al.*, 2003).

The main (if not the unique) aetiology of the disease in Spain is *B. suis* biovar 2 (Muñoz, *et al.*, 2005).

Some strains isolated in Spain keep very close genetic relationships with other *B. suis* biovar 2 strains isolated in other European countries, but most of Spanish strains have specific genetic markers, that have been only evidenced in the strains isolated in Portugal (Ferrao-Beck *et al.* 2006; Garcia-Yoldi *et al.*, 2007).

The infection due to *B. suis* biovar 2 is widely distributed among wildlife in Spain, particularly in wild boars and hares (Lavin *et al.*, 2006; Muñoz *et al.*, 2008). The prevalence of this infection in wild boars is very high varying from 11% in Asturias to over 40% in Castile la Mancha (Muñoz *et al.*, 2008).

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UNITED KINGDOM OF GREAT BRITAIN AND NORTHERN IRELAND

The UK is regarded as being free from *B. suis* infection. *B. suis* has never been recorded in animals or hares in Great Britain or Northern Ireland.

Evidence that *B. suis* remains absent from pig herds in England and Wales takes the form of monitoring animals which show clinical signs of brucellosis, for example, abortion, infertility and lameness. Samples are taken for culture and serum for antibody activity. Surveillance for *B. suis* is also required for international trade purposes. Boars intended to be used as donors for AI are also tested. So far, no *B. suis* was isolated under the surveillance initiative to provide evidence that pig herds remain free from the infection.

There is an estimated total of 4.7 million pigs in the UK with 495,000 of these being breeding pigs and 17,000 being boars used for service (June Survey of Agriculture and Horticulture, 2008). The majority of the UK breeding pig population is in England (82%) with 9% in Scotland and 1% in Wales. 92% of pigs are kept on commercial holdings and there are 1,400 such premises known. The remaining 8% of pigs are in small holdings, are estimated at 10,000. The average herd size is 500 breeding sows (EFRA select). The average breeding herd size in Wales is significantly smaller, 25 pigs per herd (Farming Facts and Figures, Wales, 2008). It is estimated that 26% of British sows are bred outdoors, however only 5% of pigs spend the growing period outdoors and 1% are finished outdoors (Assured British Pigs survey, BPEX, 2008).

Commercial farming of wild boar in the UK began in the 1980's. It was estimated that in 2004 there were 100 holdings farming wild boar with a total of 2,800 breeding sows (Figure 17). Herd size ranged from less than 10 to over 130 (Wilson, 2005). All meat produced from these holdings is subject to the same meat hygiene practices as domestic pigs.

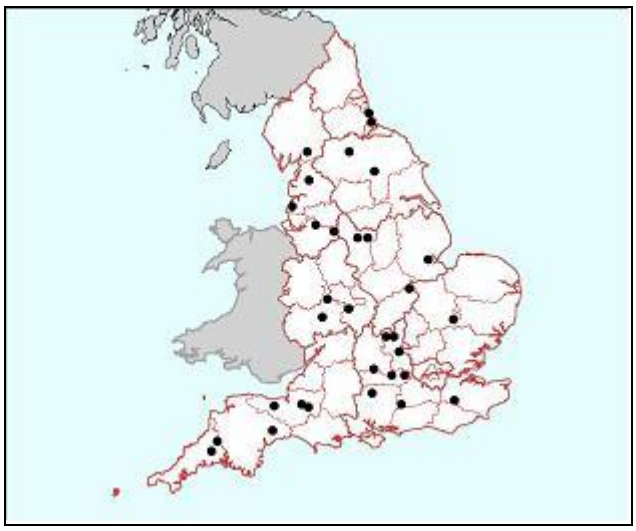

Figure 17. The distribution of wild boar holdings registered as members of the British Wild Boar Association in 2004.

In UK, it was estimated in 2004 that there were around 500 non-farmed wild boar in established wild populations in England and no more than 1000 wild boar roaming free in total. The three main populations in England are Kent/Sussex (100-200 wild boar), Forest of Dean (around 50 wild boars) and Dorset (20-30). (Moore N., 2004; Wilson C.J. 2003; Wilson

C.J. 2006) There is no data about wild boars roaming free in Scotland and Wales. It is an offence in the UK to release wild boar into the wild (Wildlife and Countryside Act, 1981).

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BULGARIA

Domestic pig population in Bulgaria

The whole domestic pig population in Bulgaria was accounted at 754,000 animals in 2007 (Table 11).

Table 11. Domestic pig population in Bulgaria for the period 2002- 2007.

	Year					
Categories of pigs	**2002**	**2003**	**2004**	**2005**	**2006**	**2007**
Slaughtered in slaughterhouses	460,500	744,500	621,900	533,300	560,000	na
Sows	73,300	76,900	78,600	79,000	81,700	70,400
Boars	na	4,620	4,669	4,511	4,420	3,920

In 2006, an Ordinance on veterinary-sanitary requirements for animal holdings was adopted. It bans rearing of sows and boars in backyards. The Ordinance allowed no more than 5 fattening pigs for own consumption to be reared in the backyards and no breeding animals present on the holding.

The size of pig holdings with breeding sows is presented on Table 12. For the period of 4 years the number of such holdings (with 1 to 2 breeding sows and with 3 to 9 sows) was reduced 2 to 3 times. There is a significant increase (77%) of holdings with more than 200 sows.

Table 12. Size of pig holdings with breeding sows in 2003 and in 2007.

Number of sows in one pig holding with breeding sows	Number of holdings (2003)	Number of holdings (2007)
1-2	22,507	8,661
3-9	4,594	1,693
10-49	969	536
50-199	98	100
More than 200	31	55

Table 13 presents the structure of domestic pig population in Bulgaria in 2007. Nearly 60% of pigs are reared in industrial holdings. Referring to the Program for the eradication of Classical Swine Fever in Bulgaria, which started at the beginning of 2008, there are 5 categories of pig holdings depending on bio-security measures in place to prevent the introduction of Classical Swine Fever. This classification could be also suitable for investigation and control of Brucellosis in Pigs.

Table 13. Domestic Pig population in Bulgaria in 2007.

Type of holding	Number of Holdings	Number of Pigs
Industrial holdings	76	450,577
Family holdings with bio-security measures	115	27,962
Family holdings without bio-security measures	3,134	103,847
Backyard holdings	72,603	172,060
Total	**75,928**	**754,446**

The total number of backyard holdings in 2007 is 75,928. Before 2006 when rearing of sows in the backyards was not forbidden in some villages, there were boars which were used for natural insemination of the sows of the same or neighbouring villages. This was an important way for the transmission of porcine brucellosis.

East Balkan Pigs

East Balkan Pig breed has been established 2,500 years ago. It originated from breeding between European wild boar and Mediterranean swine.

Table 14. Semi-wild East Balkan swine population in Bulgaria reared on pastures (mainly in oak forests) - 2007.

District	Number of herds	Number of pigs
Burgas	76	4,548
Varna	149	10,370
Shumen	57	4,475
Total	**282**	**19,393**

According to the Ordinance on veterinary-sanitary requirements on rearing of East-Balkan pigs (No 6/20.3.2007 of MAF) keeping of these animals is restricted to three Districts along the coast of Black Sea- Varna, Burgas and Shumen. These herds graze extensively in forests,

mainly covered with oak trees (acorn fruits) areas and they are likely to come into contact with wild boars (Table 14).

The permission to keep the animals on pastures is linked to the full time presence of pig-guard and it is allowed only during the daylight. The herds are under the clinical surveillance and blood sampling scheme according to the State Prophylactic Program and the Program for Control and Eradication of CSF in Bulgaria approved by the European Commission. The Ordinance lays down veterinary requirements for rearing of East Balkan pigs.

Semi-wild pigs

There is no definition of semi-wild pigs in Community legislation . From epidemiological point of view this type of pigs are more wild than domestic . These pigs have owner and a holding, but they spend more time outside than inside (especially in summer and autumn months), and their contact with wild pigs is very close.

Wild boar Population

The number of wild boar population in Bulgaria is about 57,000 (Table 15). Each year about 25,000 wild pigs are hunted. European wild boar (*Sus srofa* ferus) is the most prevalent type of wild pigs. There is another type of this animal (*Sus scrofa* Attila), mainly in the Central part of Northern Bulgaria.

Table 15. Wild Boar Population in Bulgaria- 2007.

Type of the hunting area	Number of animals
Hunting regions nominated by the State Forest Organization	55,347
National Parks	2,299
Total	**57,646**

Epidemiological situation and geographical distribution of Brucellosis in Pigs

Measures to control Porcine Brucellosis are included in the State Prophylactic Program for compulsory measures for the control of animal diseases, and laboratory tests are financed by the State Budget according to national legislation. The Program lays down compulsory tests for Porcine Brucellosis for all sows inseminated for the first time; boars- 2 times per year; all sows before insemination; swine with abortion or still births; bacteriological examination of aborted foetuses. Laboratory tests are performed in one Reference and 14 Regional laboratories for animal health. Diagnostic tests are in accordance with OIE Terrestrial Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2008a). There is no program for monitoring and surveillance of porcine brucellosis in wild boars and hares. Table 16 presents the results from laboratory tests for *B. suis* in Bulgaria for the period 1997-2008.

Table 16. Diagnostic tests for *B. suis* infection performed in Bulgaria for the period 1997-2008.

Year	Number of samples	Number of positive	% of positive	Affected districts	number of infected holdings
1997	70,750	113	0.16	Burgas	3
				Varna	1
				Pleven	1
				Plovdiv	1
				Yambol	2
1998	98,607	163	0.17	Burgas	3
				Varna	1
				Sliven	2
				Yambol	4
1999	62,134	600	0.97	Burgas	1
				Pazardzik	2
				Plovdiv	1
2000	34,095	85	0.75	Varna	1
				Ruse	1
				Yambol	1
2001	33,827	249	0.74	Burgas	2
				Tarnovo	1
				S. Zagora	2
2002	35,762	82	0.30	S. Zagora	3
				Burgas	1
				Pazadrzik	1
2003	28,971	22	0.08	Sliven	1
				S.Zagora	1
				Pazadzik	1
2004	27,844	-	-	-	-
2005	25,092	12	0.05	Silistra	1
2006	22,604	-	-	-	-
2007	20,332	-	-	-	-
2008	16,513	-	-	-	-
Total	**476,531**	**1,326**	**0.27**	**11 affected districts**	**39 infected holdings**

Source: Official animal health statistics of National Veterinary Service submitted monthly to the OIE

For the period of 1997- 2008 476,531 samples were tested for *B. suis* infection (0.27% positive). Infection was registered in 11 districts (the country is divided into 28 districts), mainly situated in Eastern Bulgaria (Figure 18). Four of the affected districts are situated in Central Bulgaria (Pazardzik, Plovdiv, Veliko Tarnovo and Pleven). No case of brucellosis in pigs was reported from Western Bulgaria. In 2 districts (Burgas, 10 infected holdings and Varna, 3 infected holdings) the East Balkan swine breed is reared. Other 2 district (Sliven, 3 infected holdings and Yambol, 7 infected holdings) have a common border with Burgas district and before year 2000 a small number of East Balkan pigs were reared on their territory. The most affected region is Burgas with 10 infected holdings.

All samples tested in 2004, 2006, 2007 and 2008 were negative. The most probable explanation for these results in 2006, 2007 and 2008 is the ban for rearing of sows and boars in backyard holdings and in holdings without bio-security measures. Epidemiological data and experience obtained for the last 50 years suggest that wild boars and East-Balkan pigs may play a role as reservoir for *B. suis* infection (Dimitrov *et al.*, 1977, Mineva *et al.*, 1991b).

Figure 18. Bulgaria. Districts affected by *B. suis* in the period 1997-2008.

(Green=districts with affected pigs holdings; Orange=districts where East-Balkans swine is reared).

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GERMANY

Occasional outbreaks of brucellosis in pigs have been recorded in the last five years. All involved holdings were outdoor holdings situated in the Northeast of Germany. An outbreak was registered, in 2004 and 2006and *B. suis* was detected at six holdings in 2008. Following former monitoring programs in 2008, a new program was started to update the knowledge on the prevalence of brucellosis in wild boars and hares in Mecklenburg-West Pomeranian.

The information available proves that brucellosis seems to be present in certain areas already for some decades. The intensity of infection rate may vary between the different geographical areas. *E.g.*: in the north-eastern region (Mecklenburg – West Pomeranian) from 201 samples 52 were found positive (the study was done by serology using an iELISA), that represents about 25% of the sampling size. In central Germany (Thuringia and Saxony) it was between 12 and 15%, in the south (Baden Württemberg) 140 samples were negative in a serological study using iELISA (Melzer *et al.*, 2006). In 1995/96 a total of 763 sera from hunted wild boars in Mecklenburg-West Pomeranian were tested and antibodies were detected in 22% of the investigated animals (Dahouk *et al.*, 2005). Similar situation can be reported in brown hares. In the northern part of Germany (Schleswig-Hollstein) 321 hares hunted in 1998-2000 were examined against *Brucella* spp. and all of them found negative (Frölich *et al.*, 2003).

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PORTUGAL

Pig production in Portugal

Portugal has a pig population around 2 334 000 animals distributed over 121,700 farms. From those, 39,000 are breeding farms with 324 300 females (Portugal National Statistics 2000). Pig production is concentrated into 3 regions: Lisbon and Tagus Valley (38.5%), Centre (30.4%) and Alentejo (22.2%).

Lisbon and Tagus Valley region with over 1 million animals, has the following distribution of animals by production systems: backyard (1 to 3 sows) 0.2%, family (4-19 sows) 6.2% and industrial (over 20 sows or 200 fattening pigs) 93.6% (official data of the Regional Directorate of Agriculture in 2007). Breeding females form 10% of the population, replacement females 1% and breeding males 0.4%.

The industrial production system is subdivided in (1) intensive; (2) intensive open-air; and (3) extensive. The main breeds and Large White and Landrace (over 2 million); Antejano (Iberian pig) (10 thousand, and concentrated mainly in Alentejo region) and Bizaro (2 thousand).

B.suis laboratorial diagnosis

Data on *B.suis* diagnosis are available from the NRL, LNIV. A summary of origin of samples and serological results (Rose Bengal Test) from 2000 to 2008 is presented in Table 17 for pigs and Table 18 for wild boars.

Table 17. Origin of samples and results of RBT in pig serum samples analysed at the PT NRL (LNIV)

Year	2000	2001	2002	2003	2004	2005	2006	2007	2008
Surveillance n	3090	1281	334	1449	2196	807	213	170	649
Positives	350+	94+	*20+	*94+	*305+	**91+	1+	1+	^31+
% positives	11.3%	7.3%	6.0%	6.5%	13.9%	11.3%	0.5%	0.6%	4.8%
Boars control	6	101	48	272	337	409	87	126	188
	*0	*4+	*16+	*1+	*11+	*4+	*6+	*4+	*1+
Exports live pigs	562	484	1600	266	1272	312	284	448	77
	0	*4+	136+	*94+	**75+	**10+	1+	0	0
Imports	281	148	405	131	761	251	123	199	6
	7+	0	6+	0	4+	0	2+	8+	0
Markets and faires	1253	126	731	1129	381	196	147	242	93
	0	0	0	*3+	*34+	0	0	*16+	6+
Suspected + abortion		61	35	20	61	6	0	0	5
		*9+	0	1+	0	0			0
TOTAL samples	5192	2201	3153	3267	5008	1981	854	1185	1018
	357+	111+	178+	193+	429+	105+	10+	29+	38+
% positive samples	6.9	5.0	5.7	5.9	8.6	5.3	1.2	2.5	3.7

Note: type of test: RBT; * RBT+CFT; ** only CFT; ^ cELISA

Table 18. Results of wildboar serum samples analysed at the PT NRL (LNIV)

Year	2000	2001	2002	2003	2004	2005	2006	2007	2008
Samples analysed		54	6	60					
% Positive RBT		5.6	0	38.3					
% Positive to CFT		22.2	0	30.0					

The results obtained at official veterinary laboratory of the Alentejo Region (Evora Lab), one of the most affected areas, are collected in Table 19.

The main reasons for submission of samples to the lab is the export of animals and the investigation of suspected cases. There is no seasonality for the submission of samples (only August appears with less samples submitted for diagnosis).

Table 19. Results of RBT in pig serum samples analysed in other Laboratories (Vairão and Évora)

Year	2000	2001	2002	2003	2004	2005	2006	2007	2008
Samples	3796	3339	1428	715	258	460	100		
RBT+	999	389	131	105	0	58	0		
% Positive	26.3	11.7	9.2	14.7	0	12.6	0		

Table 20 and table 21 present bacteriological results in the same period at LNIV, for pigs and wild boars, respectively.

Table 20. Bacteriology results in pigs analysed in Portugal (LNIV and other Laboratories) (tissues+foetus)

Year	2000	2001	2002	2003	2004	2005	2006	2007	2008
Animals tested	177+6	25+6	22+1	46+11	36+4	10+2	1	0	20
Positive	20	2	3	2	0	0	0	0	0
B. suis biovar 2	12	1	3	2					
B. melitensis biovar 3	7	1							

Table 21. Bacteriology results in wild boars analysed in Portugal (LNIV)

Year	2000	2001	2002	2003	2004	2005	2006	2007	2008
Animals tested		168	734					50	210
Positive		0	1					1	17
B. suis biovar 2		--	1					1	17

Very few materials are submitted for bacteriology and very little material is collected from wild boars. Biovar 2 is the most commonly identified in pigs and wild boars. There were some isolations of *B.melitensis* in pigs. No information exists on hares.

History of outbreaks

The occurrence of *B.suis* in Portugal is sporadic, with higher incidence in the Alentejo Region, related to the extensive production system of the Alentejano pig.

From 1999 to 2000, 12 outbreaks of brucellosis were identified in Alentejo, all in extensive farms of Alentejana breed or crosses. With the implementation of new national legislation in 2000 on eradication of brucellosis, farms using extensive system started to be tested but there were some problems in managing the slaughter and compensation of animals.

At present, serology is applied when clinical signs are present and brucellosis suspected.

Outbreaks outside Alentejo have been traced back to that Region and occurred in both intensive and intensive open-air production systems. Two outbreaks occurring in 1999-2000 are described (Vaz *et al.*, 2004).

Outbreak 1 (Lisbon and Tagus valley region)

- 1999 August - problems of abortion and metritis occur in a farm (without fever). Situation got worst by November-December. (Type of farm: intensive system, 90 breeding females)
- 2000 January - material was sent for analysis by the assistant veterinarian
- February 2 isolations of *B.suis* biovar 2 (or *B.melitensis*)
- Farm was isolated and an epidemiological inquire implemented
- Slaughter of positive breeding animals at the abattoir was decided, with collection of material for bacteriology
- Disease had spread within the farm and all animals were culled
- Investigation concluded that illegal trade was present and that animals came from an infected farm at Alentejo.
- Second farm was identified from a trace back list of a positive breeding farm in Alentejo. Total slaughter was also implemented in this farm.

- It was concluded that *B.melitensis* was the agent.

Outbreak 2 (Northern Region, Trás-os-Montes)

- 1999 September - increase of abortion in a farm with intensive open-air system (crossbreed Duroc + Bizara) (Type of farm: intensive open air system, 380 females; 30 Ha, 1000 m altitude)
- Foetuses as well as sera from 103 animals were sent to analysis - 98% were positive and isolation of *Brucella* was obtained
- Nov-Dec: due to the freezing of the drinking water system, animals used water heavily contaminated and a big outbreak of abortions occur
- Total slaughter was decided, and gradually implemented, followed by 6 months with no animals on farm.
- Infection was traced back to a farm in Alentejo where purchase of females took place. The farm of origin was not recognised as infected by that time.

National legislation

DL 378/99, 21/9 transposes Directive 98/99/CE and DL 244/2000, 27/9 is the national law on brucellosis eradication under the responsibility of the farmer and the Veterinary Authority. The provisions are that RBT is applied on pigs over 6 months of age with the slaughter of positive animals and compensation of farmer. Two negative tests with more than 6 weeks interval are necessary to re-qualify the herds and annual retest is necessary to maintain the free status. Total slaughter is carried out when over 20% of animals are found positive.

The national veterinary authority, DGV, issued the decrees nº88, 27/11/2002; and nº34, 30/4/2003 establishing the technical rules for implementation of DL 244 /2000. In intensive systems, surveys are based on RBT in animals over 6 months of age; in extensive systems RBT positive results should be confirmed with CFT. Epidemiological enquiry and compulsory in the case of outbreaks and serology must be repeated within 7 to 21 days. Bacteriology should be carried out on tissue samples from compulsory slaughtered animals.

Despite the existence of legislation, no systematic surveillance programme is, at present, carried out. Therefore there are no reliable data on the epidemiological situation of the country regarding swine brucellosis.

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Data provided by LNIV- INRB, IP in May 2009.

APPENDIX 2 – HARES (*LEPUS SPP.*) AND WILD BOARS (*SUS SCROFA*) IN EUROPE

Lepus europaeus (hare) has a large global range extending from Western Europe to western Siberia (Russia) and South-Western Asia (Iran). In Europe, the species is widely distributed throughout, with the exception of most of the Iberian Peninsula, Northern Fennoscandia and northern Russia. It inhabits a number of Mediterranean islands. The population in Ireland was introduced recently, and the population in the UK is a long-established naturalised population, that may originally have been introduced by the Romans (Battersby, 2005). As a game species, the European hare has widely been introduced to countries across the globe (Flux and Angermann, 1990). It is found from sea level to 2,300 m (Spitzenberger, 2002; Figure 19).

Introduced: Ireland; Sweden

Native - Presence confirmed: Albania; Austria; Belarus; Belgium; Bosnia and Herzegovina; Bulgaria; Croatia; Cyprus; Czech Republic; Denmark; Estonia; Finland; France; Germany; Greece; Hungary; Italy; Latvia; Liechtenstein; Lithuania; Luxembourg; Macedonia, the former Yugoslav Republic of; Moldova; Netherlands; Poland; Romania; Russian Federation; Serbia and Montenegro; Slovakia; Slovenia; Spain; Switzerland; Ukraine; United Kingdom

It is considered locally common in at least parts of its range, with typical population densities ranging from 0.2 to 0.7 individuals per hectare (Homolka and Zima, 1999). In western and central Europe, the species has undergone significant decline in the last 50 years (Flux and Angermann, 1990; Homolka and Zima, 1999; Battersby, 2005; Smith *et al.*, 2005), although there are indications that the population trend has stabilised in recent years in at least some countries (Battersby, 2005; J. Zima personal communication, 2006). Hunting bags suggest that populations in Finland are currently stable (H. Henttonen personal Communication, 2006). There is no information on population trends in eastern and south-eastern Europe. Population trend is stable.

A highly adaptable species, it occupies a wide variety of habitats, including grassland, steppes, open temperate woodland, arable farmland, and pastures (Flux and Angermann, 1990, Homolka and Zima, 1999). It tends to be particularly abundant in open, flat areas where cereal cultivation predominates. Dense old-growth forests are avoided (H. Henttonen personal Communication, 2006). Woodland, scrub, hedges and shelterbelts are used as cover when the species is resting (Homolka and Zima, 1999). It feeds mainly on grasses and herbaceous plants. When available, weeds and wild grasses are preferred, but where intensive agricultural practices have reduced the availability of these food sources, crop species are selected (Reichlin *et al.*, 2006). Unlike *Lepus timidus*, it does not feed on shrubs.

Life span is up to 13 years. An adult occupies a range of 300 hectares, which it may share with other hares as they are not territorially aggressive. There is little evidence to suggest that *L. europaeus* stays within a restricted home range.

(Sources of information:

http://animaldiversity.ummz.umich.edu/site/accounts/information/Lepus_europaeus.html and

http://www.bbc.co.uk/nature/wildfacts/factfiles/192.shtml)

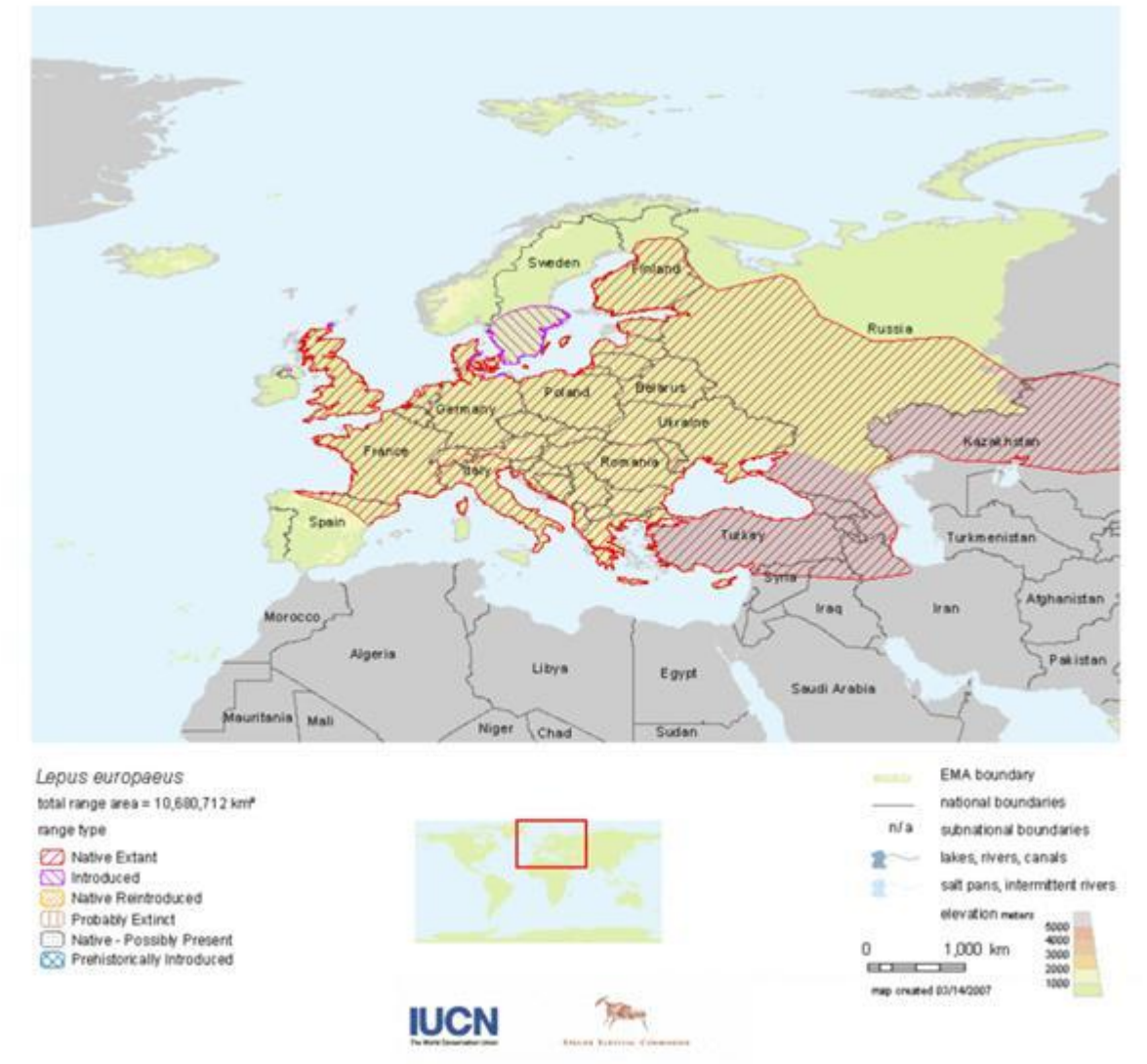

Figure 19. *Lepus europaeus*. In: IUCN 2007. European Mammal Assessment

http://ec.europa.eu/environment/nature/conservation/species/ema/species/lepus_europaeus.htm (downloaded 7.02.2009)

Lepus timidus (Mountain hare): Mountain hares range from Fennoscandia, the Baltic and east Poland to the Pacific Ocean, from 75°N in the far north of Russia and Scandinavia, south to 40-50°N. There are isolated populations in the Alps from France to Slovenia, Ireland, Scotland, Switzerland, Italy, the Kurile Islands, and Hokkaido, Japan. It has been introduced into the Faeroes, England, and various Scottish Islands; some introduced on Spitzbergen later died out. It occurs at altitudes of 250 to 3,700 m (Sulkava, 1999; Figure 20).

Introduced: Faroe Islands

Native - Presence confirmed: Austria; Belarus; Estonia; Finland; France; Germany; Ireland; Italy; Latvia; Liechtenstein; Lithuania; Norway; Poland; Russian Federation; Slovenia; Sweden; Switzerland; Ukraine; United Kingdom.

Long-term population trends in Europe appear generally stable, with fluctuations in population density occurring over a multi-year cycle (typically peaking every 4 or 7-8 years in Scandinavia and every 10 years in Scotland and northern Russia). Periodic population crashes occur, potentially as a result of disease (tularaemia, a bacterial infection), parasitism, predation, or starvation (Angerbjorn and Flux, 1995; Sulkava, 1999). Population densities of 1-10 individuals per km^2 are typical in range states (*e.g.*, Scotland and Finland). Population declines have occurred in Russia, and in the far south of Sweden the species has completely disappeared (Thulin, 2003). The isolated Alpine population may be declining (Sulkava, 1999). Population trend is stable.

Mountain hares occupy tundra and open forest, particularly of early successional stages. In Scotland and Ireland heather moors and bogland are favoured habitats, and in southern Russia copses in the middle of open steppe and reed belts around lakes. The diet varies with the habitat. In Scotland and Ireland much heather, *Calluna*, is eaten, but this is not a major food item elsewhere in Europe where willow, aspen, birch, juniper, poplar, and *Vaccinium* are favoured (Flux and Angermann, 1990). Palatable grasses and clovers are taken when available. Mountain hares are nocturnal, but there is increased daylight activity in summer when nights are short, or in winter when food is scarce (Flux and Angermann, 1990). In areas where *L. timidus* and *L. europaeus* coexist, *L. timidus* retreats to areas of higher elevation, presumably as a result of competitive exclusion (Thulin, 2003).

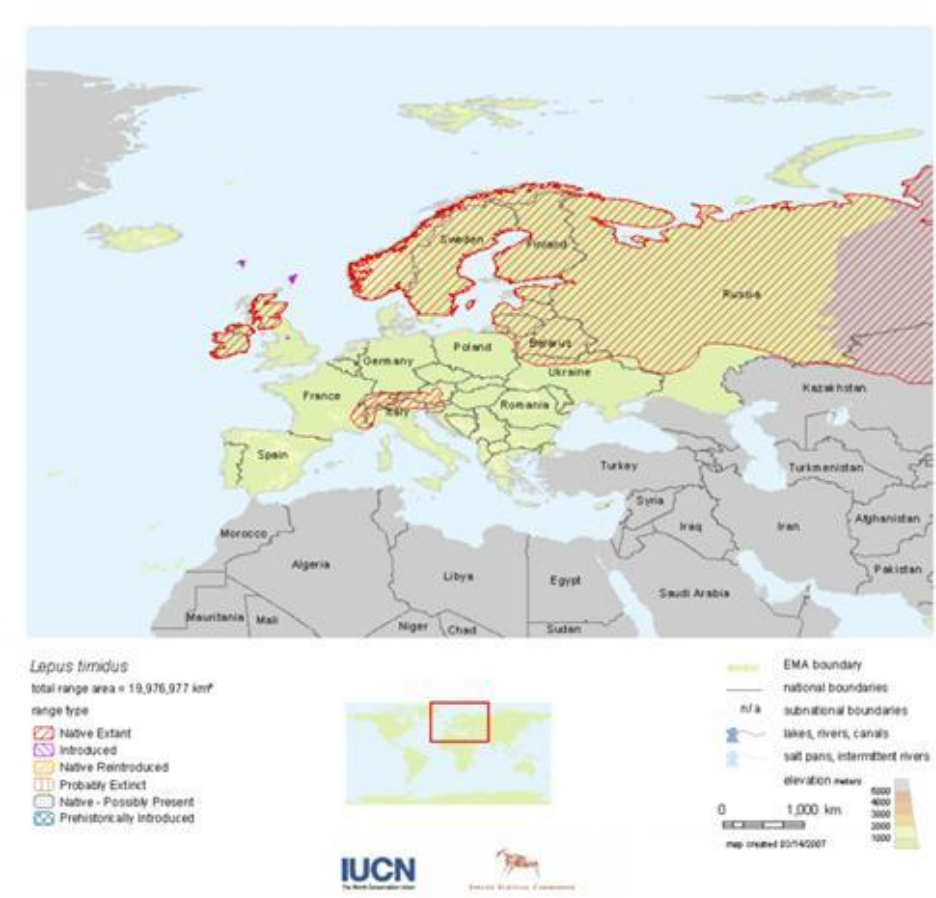

Figure 20. *Lepus timidus* In: IUCN 2007. European Mammal Assessment IUCN 2007.

http://ec.europa.eu/environment/nature/conservation/species/ema/species/lepus_timidus.htm (downloaded 7.02.2009)

Other European *Lepus* species

Lepus castroviejoi is restricted to the Cantabrian Mountains (Northern Spain) between the Sierra de Ancares and the Sierra de Peña Labra, where it occurs at altitudes from 1,000 to 1,900 m. This region is approximately 230 km from east to west and 25-40 km from north to south (Palacios, 1976; Palomo and Gisbert, 2002). The area of distribution of the species extends over approximately 5,000 km^2 (Ballesteros, 2003).

Source of information:

http://ec.europa.eu/environment/nature/conservation/species/ema/species/lepus_castroviejoi.htm

Until the 1930s, ***L. corsicanus*** was distributed in south-central Italy (the northern limit being marked by Elba Island on the Tyrrhenian coast and the province of Foggia on the Adriatic coast) and Sicily. It was also present in Corsica, where it was introduced by man in historical times (maybe between the 14^{th} and 17^{th} centuries). The current distribution of *L. corsicanus* is poorly known. In Sicily, the distribution seems to be continuous, whereas in the Italian Peninsula, populations are known only in Tuscany (in Grosseto province), Latium, Abruzzo, Molise, Apulia (Gargano), Campania, Basilicata and Calabria. As of 1984, it was considered possibly extinct in Corsica; however one dead specimen was found in 2000 and two in 2001 (Scalera and Angelici, 2003). It has been recorded from sea level to 2,400 m on Mount Etna.

Source of information:

http://ec.europa.eu/environment/nature/conservation/species/ema/species/lepus_corsicanus.htm

Lepus granatensis is endemic to Europe, being restricted to Portugal, mainland Spain, and Majorca (Spain). The population on Ibiza (Spain) has gone extinct. Attempts to introduce the species to southern France and Corsica in the last few decades of the 20th century were generally unsuccessful (Garcia-Perea and Gisbert, 1999), although a recent introduction in southern France (Perpignan) seems to have resulted in a viable population (Alves *et al.*, 2003; S. Aulagnier personal Communication, 2006). The species is reported to occur from sea level to 1,900 m (Garcia-Perea and Gisbert, 1999).

Sus scrofa (wild boar) has a large global distribution extending from western Europe and North Africa eastwards through the Middle East and central and south-east Asia, reaching its south-eastern limit at the Greater Sunda Islands. In Europe, it is widespread in most continental areas, with the exception of northern Fennoscandia and European Russia. It disappeared from the British Isles and Scandinavia in the 17^{th} century, although it has now been reintroduced to Sweden and escaped animals have established themselves in the wild in Britain (Spitz, 1999). It is native to Corsica and Sardinia, but the population in Sicily was introduced (Spitz, 1999). Animals have escaped from captivity in the UK and have established themselves in the wild. There are at least three small wild populations in England, on the Kent/East Sussex border, in Dorset, and in Hereford (Battersby, 2005). In Europe it is found from sea level to 2,400 in the Pyrenees (Palomo and Gisbert, 2002). Population trend is increasing. Wild boar populations in Europe increased markedly during the latter part of the 20^{th} century (Spitz, 1999), but are now thought to be stable in most areas (EMA Workshop, 2006). Populations in England, southern Sweden and Finland may still be increasing (Battersby, 2005; EMA Workshop, 2006; Figure 21).

Native - Presence confirmed: Albania; Austria; Belarus; Belgium; Bosnia and Herzegovina; Bulgaria; Croatia; Czech Republic; Estonia; Finland; France; Germany; Greece; Hungary; Italy; Latvia; Lithuania; Macedonia, the former Yugoslav Republic of; Moldova; Netherlands; Poland; Portugal; Romania; Russian Federation; Serbia and Montenegro; Slovakia; Slovenia; Spain; Switzerland; Turkey; Ukraine.

Reintroduced: Sweden, United Kingdom

Habitat and Ecology: It is found in a variety of temperate and tropical habitats. It prefers broadleaved forests and especially evergreen oak forests, but may also be found in more open habitats such as steppe, Mediterranean shrubland, and farmland, so long as there is water and tree cover nearby (Spitz, 1999). It has an omnivorous diet, consuming vegetable matter (*e.g.* beech mast, acorns, green plants, tubers), carrion, and live animal prey (earthworms, insect larvae, small vertebrates) (Herre, 1986, Oliver, 1993).

In Bulgaria, the number of wild boar population in Bulgaria is about 57,000 and about 25,000 wild pigs are hunted each year. The density of these pigs is about 0.5 animal per sqkm. European wild boar (*Sus scrofa* ferus) is the most wide spread type of wild pigs. There is another type of this animal- *Sus scrofa* attila, mainly in the Cental part of Northern Bulgaria. There is no information about presence of *B. suis* infection in wild boars, no laboratory tests are performed.

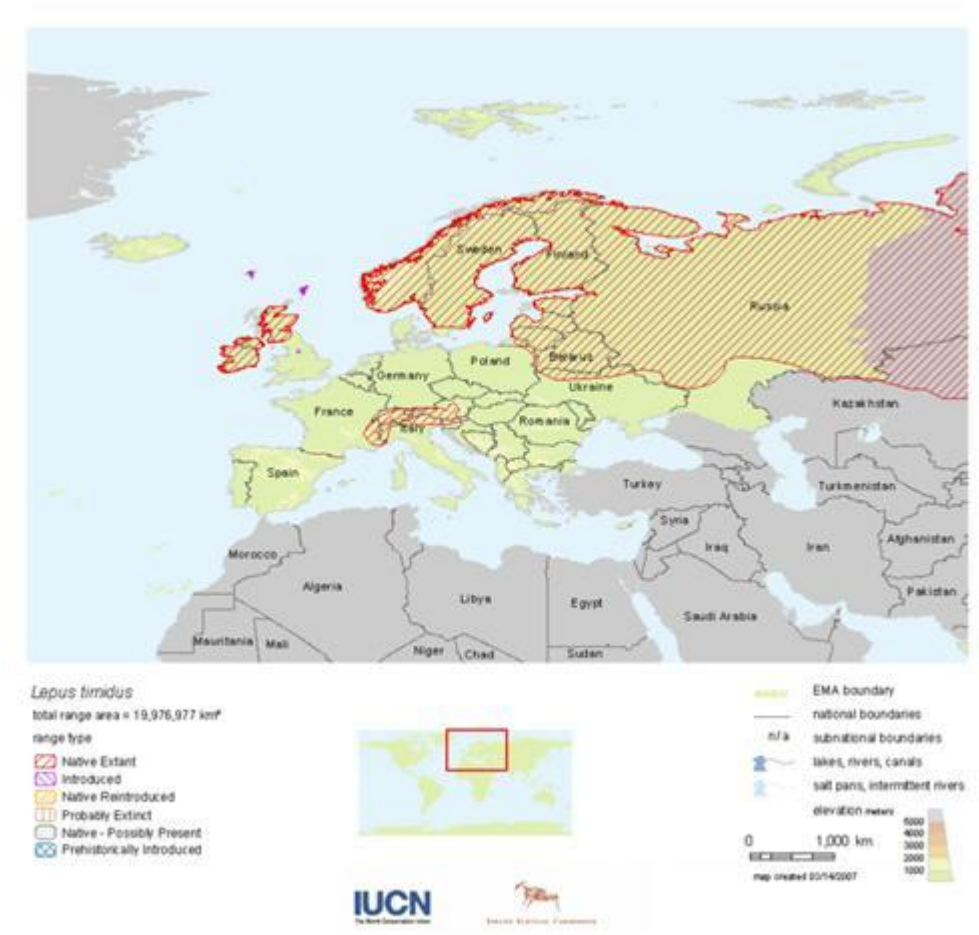

Figure 21. *Sus scrofa* In: IUCN 2007. European Mammal Assessment IUCN 2007.

http://ec.europa.eu/environment/nature/conservation/species/ema/species/sus_scrofa.htm downloaded 7.02.2009

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APPENDIX 3 – DATA REQUESTED TO NRL

European Food Safety Authority

ANNEX

Scientific studies

EFSA is interested in data from all relevant scientific studies, irrespective of the actual results. We would like to receive both published information as well as data that have not been previously published. Given your organization's role in this area, the working group of EFSA for this mandate would appreciate your assistance in identifying available reports with data from scientific studies that could be of value in addressing this request from the Commission.

We appreciate if you can include both a written description of the study as well as the raw data, both preferably in an electronic form. The reports and their data will be duly acknowledged in the opinion. However, unpublished data will not be disclosed without your written permission.

Only studies related to porcine brucellosis caused by *Brucella suis* should be included and the following elements of the study description should be described:

Materials: sampling method, sampling period, characterization of animals from which the samples were derived including animal species, status of both the herd(s) and of the individual study animals regarding brucellosis infection and brucellosis vaccination. If animals were vaccinated, both the age at which they were vaccinated and the age at which they were tested is requested.

Methods: description of all the tests that were compared (including the reference test). Please provide sufficient detail so as to be able to reproduce the test in the future. Elements to be considered are the approach used to set the cut-off and the control of kit reagents;

Statistical analysis: methods used to summarize or analyze the data;

Results: reported diagnostic sensitivity and specificity, along with sample size for each calculation.

For the raw data provided along with the study information, we propose that you use the format shown in Table 1 (attached), by animal species and for every herd separately.

Please include in your submission as part of the mail a list of the submitted documents and their references. The name and contact details of the party providing the scientific contribution, is kindly required. Unless explicitly stated otherwise by the submitting body, the working group of EFSA expects that data on all conducted studies will be provided as well as the complete information for each study. Please also note that, unless duly justified, the data will not be considered confidential, however, the company's name will not be disclosed and the test will be coded for data analysis and publication. If there is copyright on a document please indicate such information and whether the holder authorizes EFSA to reproduce, distribute, and communicate this scientific contribution.

Routine testing

EFSA is also interested in summary data regarding routine testing in geographical areas (either the entire Member States or areas within a Member State) in which all pig herds are officially free of brucellosis. The rate and the nature of the false positive results obtained should help characterize test specificity at the level of the individual animal. If you provide the raw data on this we propose that you utilise Table 2 (attached).

Confirmed cases

If relevant, and based on the information you might request from your National Authorities, EFSA would be grateful if you could also provide information about the sources of infection in confirmed cases of *Brucella suis* in domestic pigs in your country during 2007 and 2008 as well as the results of diagnostic studies about Brucellosis in pigs, for which we propose that you use the format shown in Table 3 (attached).

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Table 1. Test results for brucellosis diagnostic test conducted in pigs (not Wild Boars).

if similar data are available in different format (i.e. in electronic form), you may submit them in your original format.

Herd data:

Herd ID_____

Brucellosis infection status: ☐ infected ☐ free since_____(Month, Year)

Individual animals received confirmatory test? ☐ Yes, method: _____ ☐ No

Does the sample below include multiple results form the same animal: ☐ Yes ☐ No

Animal ID*	Age at bleeding**	RBT	CFT	iELISA	cELISA	FPA	TAT	Skin test	Gamma-interferon test	Other (enter name of test)	Confirmatory test result***

*not strictly required but appreciated when available.

** s=sow, b=boar, f=finisher, p=piglet

*** please provide code for positive, negative and missing results.

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Table 2. Test results from countries or areas with only pig herds that are officially brucellosis free.

Year: _____

Region or Country:_____

	RBT	CFT	iELISA	cELISA	FPA	TAT	Skin test	Gamma-interferon test	Other (enter name of test)
Number tested									
Number of positive results									
Number of intermediate results (if applicable)*									

*enter NA for not applicable or number of results: Zero (0) means that the test has an intermediate range but no outcomes were observed in this category.

Please state if positive test results of preliminary tests (e.g., screening) were ruled-out or confirmed by subsequent tests. In this case please describe the sequence of tests.

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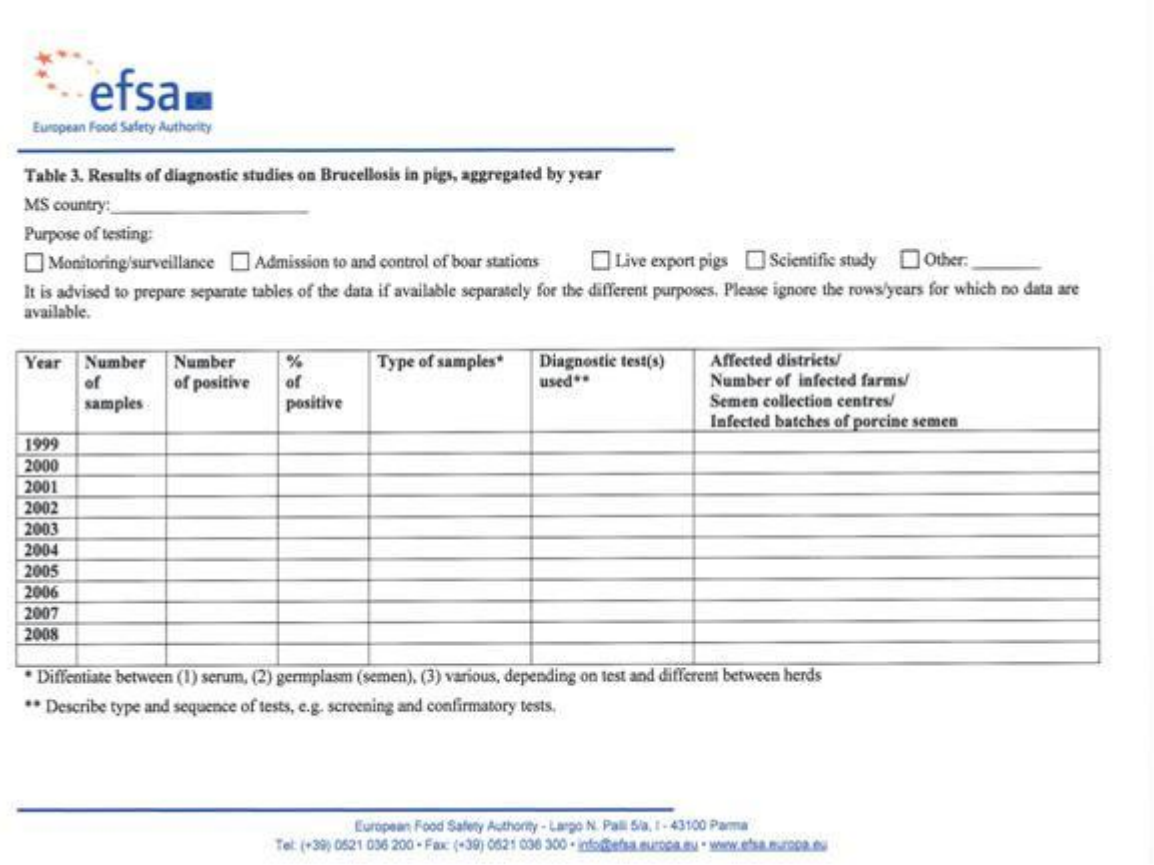

Table 3. Results of diagnostic studies on Brucellosis in pigs, aggregated by year

MS country:_____

Purpose of testing:

☐ Monitoring/surveillance ☐ Admission to and control of boar stations ☐ Live export pigs ☐ Scientific study ☐ Other: _____

It is advised to prepare separate tables of the data if available separately for the different purposes. Please ignore the rows/years for which no data are available.

Year	Number of samples	Number of positive	% of positive	Type of samples*	Diagnostic test(s) used**	Affected districts/ Number of infected farms/ Semen collection centres/ Infected batches of porcine semen
1999						
2000						
2001						
2002						
2003						
2004						
2005						
2006						
2007						
2008						

* Diffentiate between (1) serum, (2) germplasm (semen), (3) various, depending on test and different between herds

** Describe type and sequence of tests, e.g. screening and confirmatory tests.

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Note: A similar questionnaire was sent ot the Veterinary Diagnostic Companies.

APPENDIX 4 – DATA FROM NATIONAL REFERENCE LABORATORIES. NUMBER OF REACTORS ON DOMESTIC PIG SERUM SAMPLES TESTED[1]

Country	Year	RBT	CFT	iELISA	FPA	SAT	Skin Test	γ-interferon test
Bulgaria	2008	0/20170	0/20170					
Denmark	2003		36/39			39/200	0/39	0/39
Denmark	2006	7/16068	0/4043			5/2928		
Denmark	2007	9/17443	12/4841			9/2311		
Denmark	2008	28/15949	95/6118			38/2729		
Estonia	2005	0/1562						
Estonia	2006	1/1517	0/4					
Estonia	2007	0/1134						
Estonia	2008	0/4004						
Estonia	NA	2/1407	0/3					
Finland	2008	27/3330	0/27					
Ireland	2007	8/1529	0/8			8/8		
Ireland	2008	1/1657	0/2			1/2		
Latvia	2007	2/6266	/15					
Latvia	2008	14/9652	/37					
Poland	2000	3/2487	1/159	3/2478		0/293		
Poland	2001	3/6070	0/3231	0/6194		1/3388		
Poland	2002	4/5635	0/778	0/8463		0/838		
Poland	2003	12/7001	1/1141	2/7633		6/1604		
Poland	2004	4/8763	0/289	2/10050		0/299		
Poland	2005	16/8045	0/343	7/16949		2/240		
Poland	2006	30/14568		0/24183				
Poland	2007	6/13820		0/37035				
Poland	2008	26/5087	9/46	25/12090		12/46		
Slovak R.	2008	0/1390	0/5094					
Sweden	1999					0/3000		
Sweden	2000					0/3000		
Sweden	2001					0/3000		
Sweden	2002					0/3000		
Sweden	2003					0/3000		
Sweden	2004					0/3000		
Sweden	2005	0/3000						
Sweden	2006	0/3000						
Sweden	2007	0/3000						
Sweden	2008							
United Kingdom	NA	978/27193	509/9797			1210/1406 2		
France[2]		122/4623		74/4623	197/4623			
France[3]		9/901		7/901	1/901			

[1] The data suggest not all MS reported the results including False Positive Serological Reactions (FPSR).
[2] Officially brucellosis free herds in metropolitan France
[3] Herds considered as brucellosis free in French Polynesia

APPENDIX 5 – TEMPLATE FOR DATA ENTRY

EFSA Brucellosis/pigs WG— Data collection form for systematic review of diagnostic tests

Paper (0) RefID:	(1) Paper Identity:			(3) ☐ Of interest for chapter(s)	
	(2) ExCode:			(4) ☐ Multiple tests compared	
Diagnostic test(s)					
(5) Name of test #1: T1 (6)	☐ BPAT ☐ CFT ☐ SAT ☐ ELISA ☐ cELISA ☐ FPA ☐ Skin ☐ RBT ☐ PCR other:				
(5) Name of test #2: T2 (6)	☐ BPAT ☐ CFT ☐ SAT ☐ ELISA ☐ cELISA ☐ FPA ☐ Skin ☐ RBT ☐ PCR other:				
(5) Name of test #3: T3 (6)	☐ BPAT ☐ CFT ☐ SAT ☐ ELISA ☐ cELISA ☐ FPA ☐ Skin ☐ RBT ☐ PCR other:				
(5) Name of test #4: T4 (6)	☐ BPAT ☐ CFT ☐ SAT ☐ ELISA ☐ cELISA ☐ FPA ☐ Skin ☐ RBT ☐ PCR other:				
(5) Name of test #5: T5 (6)	☐ BPAT ☐ CFT ☐ SAT ☐ ELISA ☐ cELISA ☐ FPA ☐ Skin ☐ RBT ☐ PCR other:				
(7)...(11) additional data for test #1	☐ standardized ☐ cyto ☐ outer ☐ other antigen ☐ Com (12) ExCode:				
(7)...(11) additional data for test #2	☐ standardized ☐ cyto ☐ outer ☐ other antigen ☐ Com (12) ExCode:				
(7)...(11) additional data for test #3	☐ standardized ☐ cyto ☐ outer ☐ other antigen ☐ Com (12) ExCode:				
(7)...(11) additional data for test #4	☐ standardized ☐ cyto ☐ outer ☐ other antigen ☐ Com (12) ExCode:				
(7)...(11) additional data for test #5	☐ standardized ☐ cyto ☐ outer ☐ other antigen ☐ Com (12) ExCode:				
Reference populations (RefPop)	RefPop 1	RefPop 2	RefPop 3	RefPop 4	RefPop 5
(13) Short name	Name R1	Name R2	Name R3	Name R4	Name R5
(14) Reason for exclusion (code), or ...	ExCode:	ExCode:	ExCode:	ExCode:	ExCode:
(15) Parameter	☐ Se ☐ Sp	☐ Se ☐ Sp	☐ Se ☐ Sp	☐ Se ☐ Sp	☐ Se ☐ Sp
(16) Study type	☐ Epi ☐ Exp	☐ Epi ☐ Exp	☐ Epi ☐ Exp	☐ Epi ☐ Exp	☐ Epi ☐ Exp
(17) Infection status (applies to Se+Epi)	☐ acute ☐ end	☐ acute ☐ end	☐ acute ☐ end	☐ acute ☐ end	☐ acute ☐ end
(18) Area of origin	☐ EU ☐ other	☐ EU ☐ other	☐ EU ☐ other	☐ EU ☐ other	☐ EU ☐ other
(19) Gold standard criterion	☐ bact ☐ other	☐ bact ☐ other	☐ bact ☐ other	☐ bact ☐ other	☐ bact ☐ other
(20) Individual (versus herd) confirmation?	☐ ind ☐ herd	☐ ind ☐ herd	☐ ind ☐ herd	☐ ind ☐ herd	☐ ind ☐ herd
(21) Known Yersinia status (applies to Sp)	☐ pos ☐ neg	☐ pos ☐ neg	☐ pos ☐ neg	☐ pos ☐ neg	☐ pos ☐ neg
(22) Days post infection (applies to Se+Exp)	dpi:	dpi:	dpi:	dpi:	dpi:
Validation results Test #1 (23)	ExCode:	ExCode:	ExCode:	ExCode:	ExCode:
(24) Page number and/or table number	Tab.	Tab.	Tab.	Tab.	Tab.
(25) Number of true results					
(26) Number of inconclusive results	if any	if any	if any	if any	if any
(27) Sample size					
(28) Parameter (percent Se or Sp)	no comma	no comma	no comma	no comma	no comma
Validation results Test #2 (23)	ExCode:	ExCode:	ExCode:	ExCode:	ExCode:
(24) Page number and/or table number	Tab.	Tab.	Tab.	Tab.	Tab.
(25) Number of true results					
(26) Number of inconclusive results	if any	if any	if any	if any	if any
(27) Sample size					
(28) Parameter (percent Se or Sp)	no comma	no comma	no comma	no comma	no comma
Validation results Test #3 (23)	ExCode:	ExCode:	ExCode:	ExCode:	ExCode:
(24) Page number and/or table number	Tab.	Tab.	Tab.	Tab.	Tab.
(25) Number of true results					
(26) Number of inconclusive results	if any	if any	if any	if any	if any
(27) Sample size					
(28) Parameter (percent Se or Sp)	no comma	no comma	no comma	no comma	no comma
Validation results Test #4 (23)	ExCode:	ExCode:	ExCode:	ExCode:	ExCode:
(24) Page number and/or table number	Tab.	Tab.	Tab.	Tab.	Tab.
(25) Number of true results					
(26) Number of inconclusive results	if any	if any	if any	if any	if any
(27) Sample size					
(28) Parameter (percent Se or Sp)	no comma	no comma	no comma	no comma	no comma
Validation results Test #5 (23)	ExCode:	ExCode:	ExCode:	ExCode:	ExCode:
(24) Page number and/or table number	Tab.	Tab.	Tab.	Tab.	Tab.
(25) Number of true results					
(26) Number of inconclusive results	if any	if any	if any	if any	if any
(27) Sample size					
(28) Parameter (percent Se or Sp)	no comma	no comma	no comma	no comma	no comma
(29) **Comments**					

Workflow: ☐ 1st review completed ☐ 2nd review completed ☐ full agreement ☐ disagreement resolved ☐ disagreement not resolved ☐ Data released (2nd reviewer) ☐ Data released (EFSA)

APPENDIX 6 – DATA COLLECTION FORM FOR SYSTEMATIC REVIEW OF DIAGNOSTIC TESTS - CODES FOR DATA ANALYSIS

(0) RefID	Enter Reference identification number (integer between 1 and 1092)
(1) Paper Identity	Identification of the paper. Enter as "FirstAuthorNameYYYYabc", No space, No special symbols! Examples: Smith2000a, Smith2000b
(2) ExCode	Exclusion code applicable to the whole paper : 1=not about the subject (validation of Bruc tests in pigs) 2=not an original report (e.g. duplicated publication or review paper 3=(this level is not in use; note that publication date is no exclusion criterion) 4=no translation available (Check first with WG) 14=failure on multiple criteria
(3) Of interest for..	Tick-the-box if paper is relevant for another chapter of the report. Enter chapter for which paper is of interest (separate by semicolon), 2=agent, 3=epi, 4=patho, 5=clin, 6=diag, 8=risk factors, 9=control. Box may be ticked if paper is excluded.
(4) Multiple tests	Tick-the-box if the paper contains cross-tabulated results of 2 (or more) relevant tests in 2 (or more) populations or if latent class analysis was used to estimate Se and Sp of tests. Box may be ticked if Se and Sp estimates are excluded.
(5) Name of test	Enter clear and brief name of test (max. 12 letters). Repeat for up to 5 tests. Use new sheet for more than 5 tests.
(6) Test principle	Tick-the-box for the appropriate test principle (exactly 1 box per line must be ticked for each valid test). BPAT Brucella plate agglutination test CFT Complement fixation test SAT Serum agglutination test iELISA indirect Enzyme-linked immunosorbent assay cELISA Competitive enzyme-linked immunosorbent assay FPA Fluorescence polarisation assay Skin Brucellin allergic skin test RBT Rose Bengal test PCR Polymerase chain reaction other other test principle (enter short name)
(7) standardized	Tick-the-box if antigen and whole method is standardised according to OIE, EU or other (applies to CFT, SAT, cELISA, RBT)
(8) cyto	Tick-the-box if cytosolic fraction used as test antigen
(9) outer	Tick-the-box if outer membrane proteins used as test antigen
(10) other antigen	Tick-the-box if any other antigen including whole cell, any LPS preparation, OPS
(11) Com	Tick-the-box if reagents or kit is/was commercially available
(12) ExCode	Exclusion code applicable to one particular test: 5=test is not relevant (add brief justification) 6= (this level is not in use) 7=test described insufficiently, no judgement possible 14=failure on multiple criteria
(13) Short name	Enter clear and brief name of reference population (RefPop) as used in the paper (max.15 letters). Use new sheet for more than 5
(14) ExCode	Exclusion code applicable to one particular RefPop: 8=animals are not domestic pigs (level may be use to flag borderline cases) 9=animals are vaccinated against Brucella 10=experimental infection with Yersinia or selective serum panel for Yers. cross-reactions 11= gold standard is inappropriate (e.g. new test is part of the "gold standard") 14=failure on multiple criteria
(15) Parameter	Se: Tick-the-box if Brucella infection is considered present in the population and sample is used to estimate sensitivity Sp: Tick-the-box if Brucella infection is considered absent from the population and sample is used to estimate specificity
(16) Study type	Epi (epidemiological study): Tick-the-box if the sample was selected from some natural population of domestic pigs Exp (experimental study): Tick-the-box if the sample was taken from swine experimentally infected with B. suis
(17) Infection status	acute: Tick-the-box for recent outbreak (typically multiple clinical cases in herd) end: Tick-the-box for endemic situation (typically no clinical signs or herd is known to be infected since some time) Note: one of these boxes should only be ticked if Se and Epi was also ticked.
(18) Area of origin	EU: Tick-the-box if sample is from any of the EU27 MS plus Switzerland and Norway other: Tick-the-box if sample is from any other area
(19) Gold standard	bact: Tick-the-box if bacteriological culture was used as gold standard or part of gold standard other: Tick-the-box if other test results or other criteria not including bacteriology were used Note: none of these boxes should be ticked if no tests were conducted to characterise the reference population
(20) Confirmation	ind: Tick-the-box if all individual animals used for calculations are confirmed using the gold standard test herd: Tick-the-box if not all individual animals used for calculations have received the gold standard test Note: none of these boxes should be ticked if no tests were conducted to characterise the reference population
(21) Yersinia status	pos: Tick-the-box if at least some animals in the sample are diagnosed Yersinia enterocolitica serotype 0.9 positive neg: Tick-the-box if Yersinia enterocolitica serotype 0.9 is not confirmed or status is unknown Note: these boxes should only be ticked if Parameter = Sp
(22) Days post inf.	Enter mean number of days post infection (dpi) if experimental study was used. Notes: Select only 2 Se estimates per test which are closest to dpi = 30 days (acute) and dpi = 120 days (chronic). Animals will be mostly) identical and tested at two time points. Report the results as 2 reference populations (RefPops). The names of the 2 RefPops should be identical. These boxes should only be ticked if Parameter = Se and and Study = Exp.
(23) ExCode	Exclusion code applicable to one particular estimation of Se or Sp: 12=no way to read or calculate diagnostic Se or Sp (for example, results in tables and text are contradicting) 13 =no sample size given 14=failure on multiple criteria
(24) Page/Table	Enter page(p) or Table (T) number from where the result has been read.
(25) Correct results	Enter number of correct positive (negative) test results for estimation of Se (Sp).This field may not be empty.
(26) Inconclusive	Enter number of non-positive non-negative test results (intermediate zone). Note: Enter "0" if inconclusive results are valid but were not reported. Leave blank if valid inconclusive results cannot occur.
(27) Sample size	Enter number of infected (non-infected) animals used for estimation of Se (Sp). This field may not be empty.
(28) Parameter	Enter percent value Se or Sp with one decimal, eg 95.2. Note: Use the point and NOT THE COMMA as decimal sign. This value is used for plausibility control only.
(29) Comment	Enter comments about potential biases, etc. This data field will not be used in analysis.

APPENDIX 7 – WORKFLOW FOR CONDUCTING THE LITERATURE REVIEW

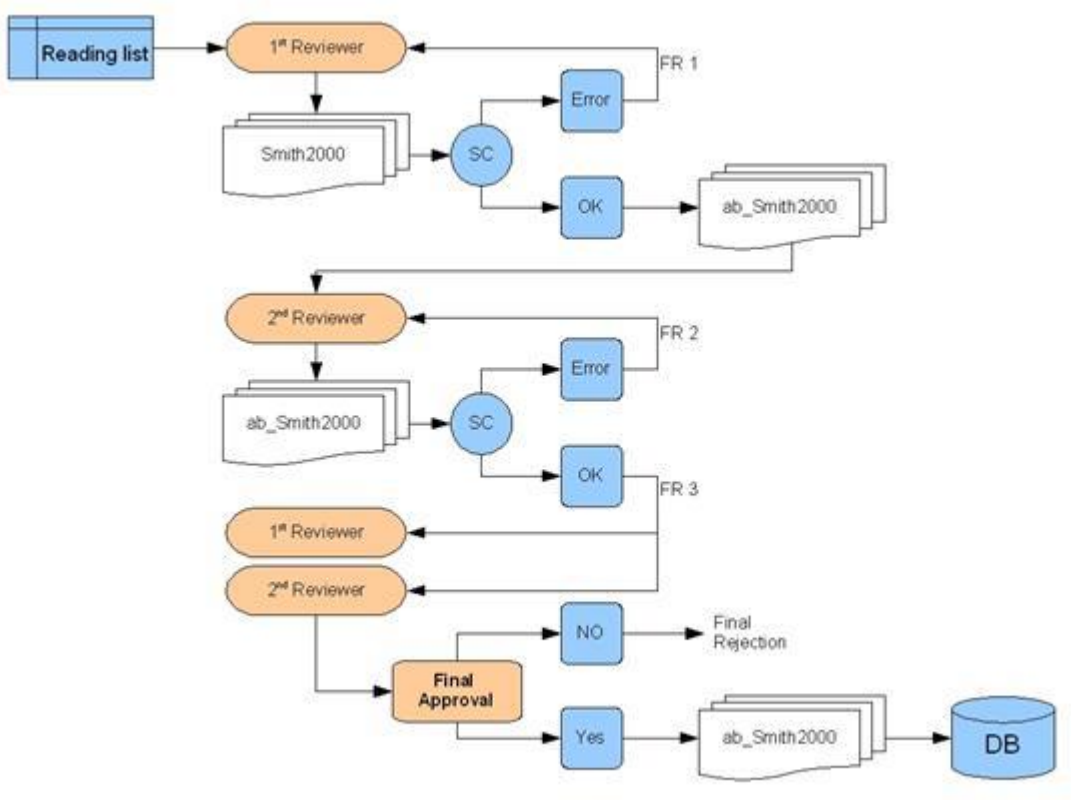

Figure 22. Sequential workflow for conducting the literature review based on full papers (stage 2) with random allocation of a 1st and 2nd reviewer to each paper.

1st reviewer collected data, any non-plausible entries were reported back (FR1) while plausible data sheets were forwarded by study centre (SC) to 2nd reviewer. After passing further plausibility test (FR2), identity of 1st and 2nd reviewer was disclosed and 2nd reviewer was responsible for resolution of any discrepancies. Final approval (involving study if required) resulted in rejection (did not occur) or acceptance into study data base (DB).

APPENDIX 8 – LIST OF SCIENTIFIC PUBLICATIONS INCLUDED IN THE FINAL ANALYSIS

Abdoel T., Dias I. T., Cardoso R., Smits, H.L. (2008). Simple and rapid field tests for brucellosis in livestock. Veterinary Microbiology, 130: 312-319.

Becker H.N., Belden R.C., Breault T., Burridge M.J., Frankenberger W.B., Nicoletti P. (1978). Brucellosis in feral swine in Florida, Journal of the American Veterinary Medical Association. 173: 1181-1182.

Dedie K., Lehnert C., Jendrusch H. (1958). Intradermal allergic tests for diagnosis of brucellosis in pigs. Archiv für experimentelle Veterinärmedizin. 12: 193-201.

Ferris R.A., Schoenbaum M. A., Crawford R. P. (1995) Comparison of serologic tests and bacteriologic culture for detection of brucellosis in swine from naturally infected herds. Journal of the American Veterinary Medical Association. 207: 1332-1333.

Nielsen K., Smith P., Yu W., Nicoletti P., Elzer P., Vigliocco A., Silva P., Bermudez R., Renteria T., Moreno F., Ruiz A., Massengill C., Muenks Q., Kenny K., Tollersrud T., Samartino L., Conde S., Benite, G.D.D., Gall D., Perez B., Rojas X. (2004). Enzyme immunoassay for the diagnosis of brucellosis: chimeric Protein A-Protein G as a common enzyme labeled detection reagent for sera for different animal species. Veterinary Microbiology, 101: 123-129.

Nielsen K., Smith P., Yu W., Nicoletti P., Jungersen G., Stack J., Godfroid J. (2006). Serological discrimination by indirect enzyme immunoassay between the antibody response to *Brucella spp*. and *Yersinia enterocolitica* O:9 in cattle and pigs. Veterinary Immunology and Immunopathology, 109: 69-78.

Ortiz E., Nibot C., Silva E., Izquierdo M., Cabrera C., Rodriguez O. (2005). Application of DAVIH BRU 3 ELISA system in the serological diagnosis of pig brucellosis. Revista de Salud Animal, 27: 166-170.

Riber U. and Jungersen G. (2007). Cell-mediated immune responses differentiate infections with *Brucella suis* from Yersinia enterocolitica serotype O:9 in pig. Veterinary Immunology and Immunopathology, 116: 13-25.

Szulowski K. and Pilaszek J. (2001). Current aspects of brucellosis diagnosis in wild animals, Medycyna Weterynaryjna, 57: 867-869

Szulowski K. (1999. Diagnosis of *Brucella suis* infections in pigs and hares by ELISA. Polish Journal of Veterinary Sciences, 2: 65-70.

Szulowski K., Pilaszek J., Truszczynski M. (1996). An ELISA kit for the examination of swine sera for brucellosis. Medycyna Weterynaryjna, 52: 513-515.

Thirlwall R.E., Commander N.J., Brew S.D., Cutler S.J., Mcgiven J.A., Stack J.A. (2008). Improving the specificity of immunodiagnosis for porcine brucellosis, Veterinary research communications, 32: 209-213.

Van Der Giessen J.W. and Priadi A. (1988). Swine brucellosis in Indonesia, Veterinary Quarterly, 10: 172-176.

APPENDIX 9 – META-ANALYSIS MODEL

The following model was used for meta-analysis of diagnostic tests using Bayesian logistic regression (BRugs package for R, Andrew *et al.*, 2006)

```
model {
        beta0  ~ dnorm(0.0,1.0E-6)I(-15,15)
        beta1  ~ dnorm(0.0,1.0E-6)I(-15,15)
        for (she in 1 : N ) {
                logit(p[i]) <- beta0 + beta1*x[i]
                k[i] ~ dbin(p[i],n[i])
                }
                theta <- 1/(1+exp(-(beta0)))
        }
```

where beta0 and beta1 are model coefficients, dnorm refers to Normal variate with mean and precision (squared inverse standard error), I(min,max) denotes a truncation interval, N denotes the number of primary estimates of the parameter p (Se or Sp), x, k and denote the observed indicator variable, number of true results and sample size of the ith primary estimate, respectively, dbin denotes a binomially distributed variate and theta is the posterior estimate of the parameter p predicted for the level x=0 calculated using inverse logit function.

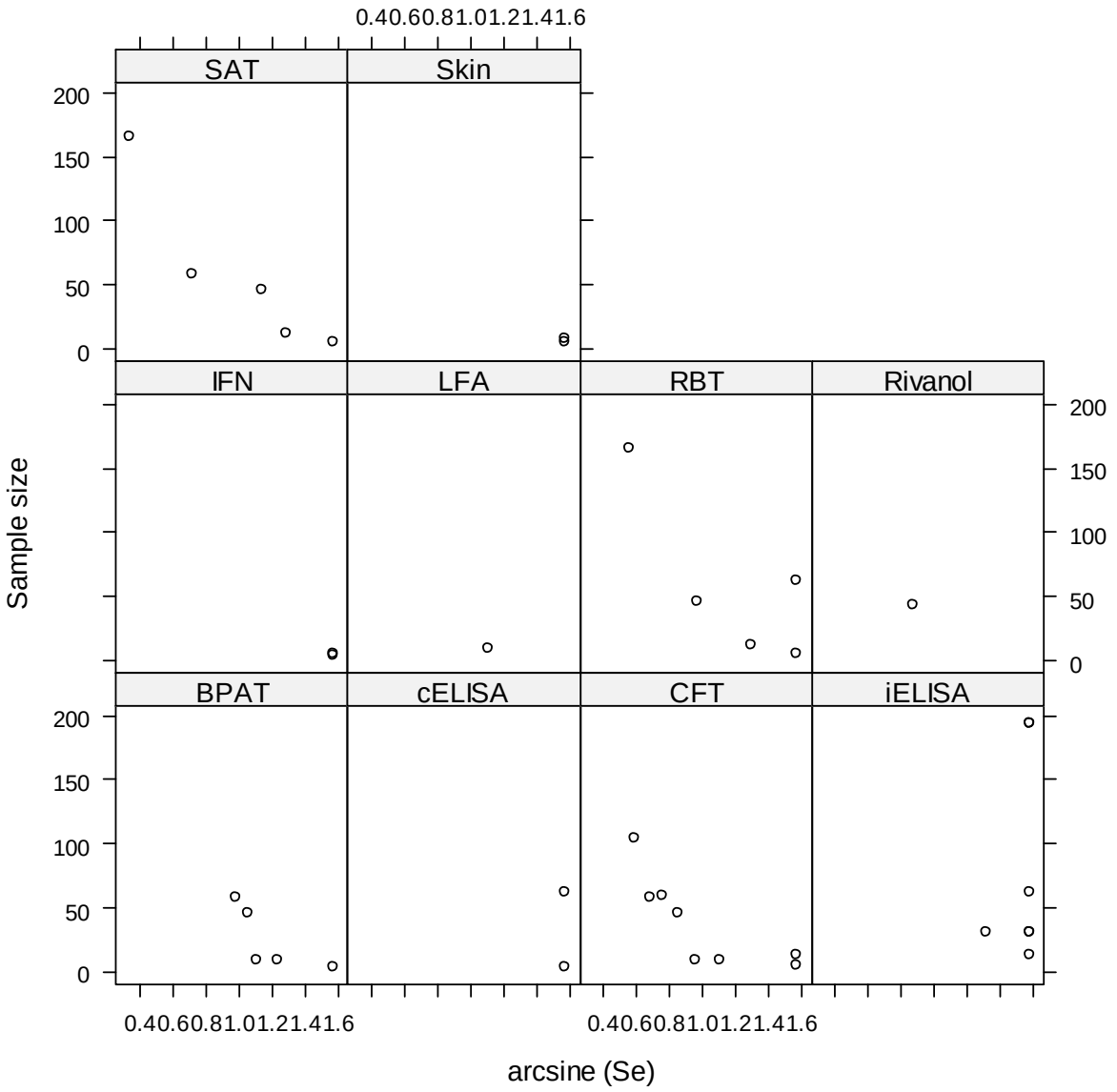

Figure 23. Funnel plots for sensitivity (Se) for diagnostic tests for Brucellosis in pigs to explore publication bias.

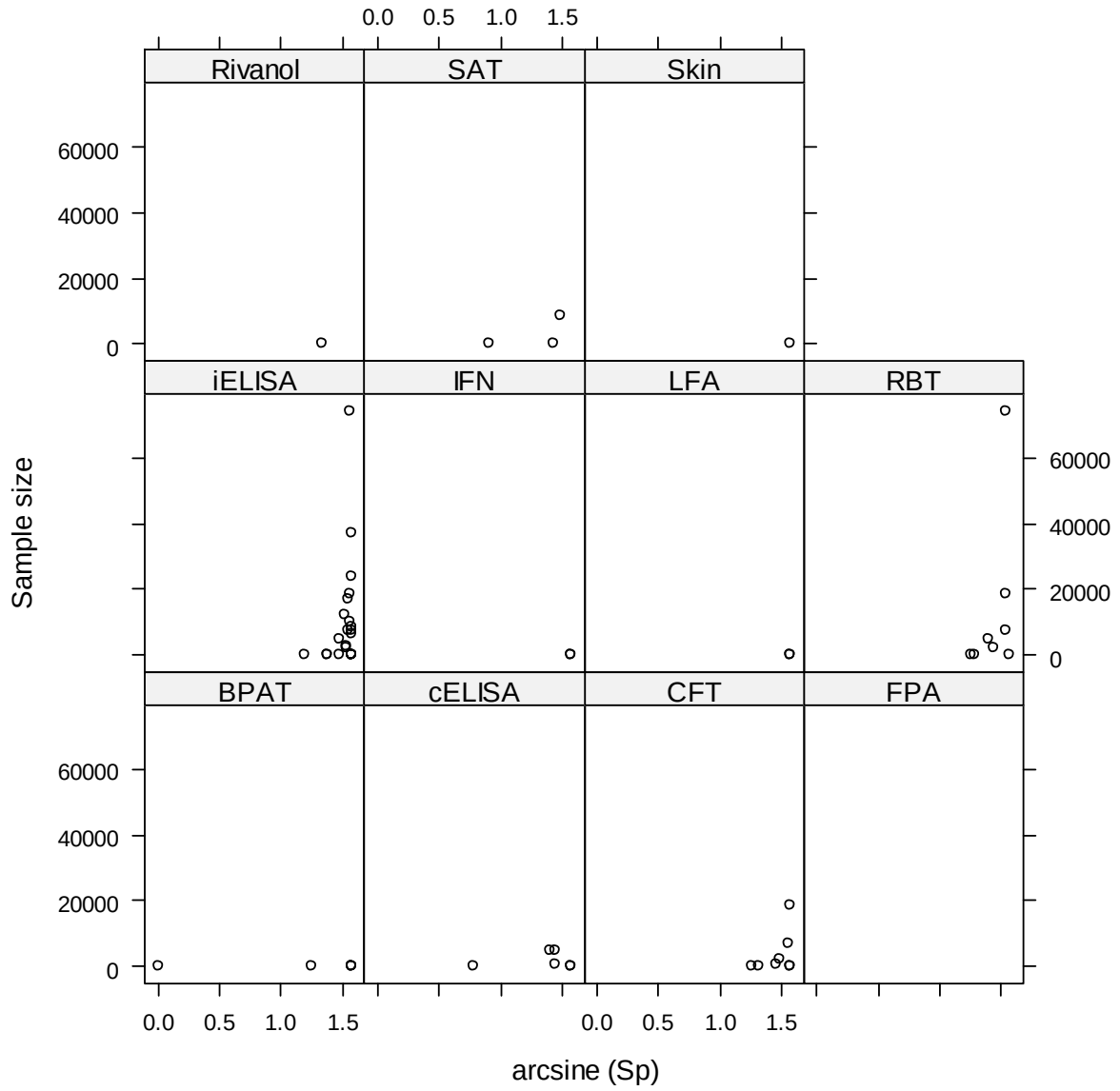

Figure 24. Funnel plots for specificity (Sp) for diagnostic tests for Brucellosis in pigs to explore publication bias.

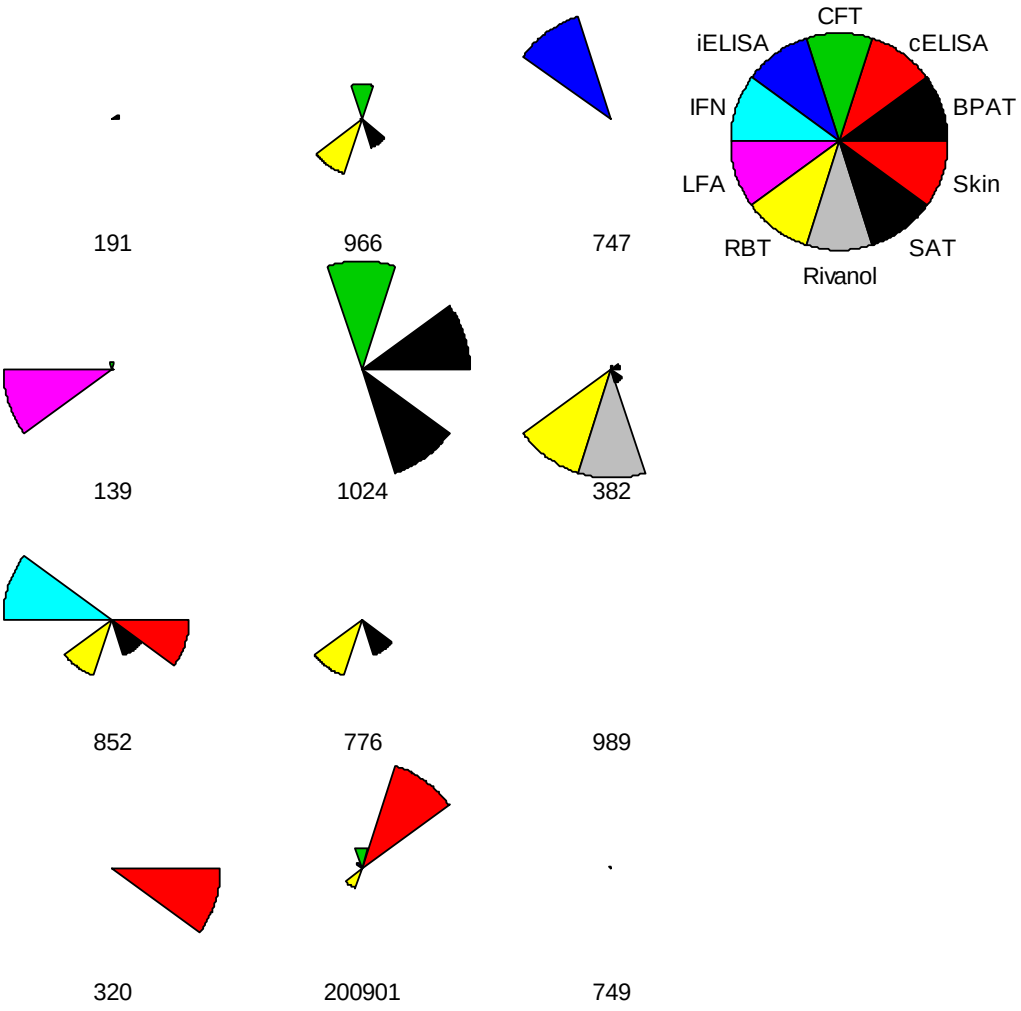

Figure 25. Impact of references on point estimates of Se - "Leverage plot".

"Leverage plot" consisting of one pie chart for each source paper (reference ID shown below each pie chart) with sizes of slices proportional to the impact of the study on the meta-analysis summary estimate of sensitivity (Se) of the respective test. Pie chart in upper right corner shows colour legend for diagnostic tests evaluated for Se.

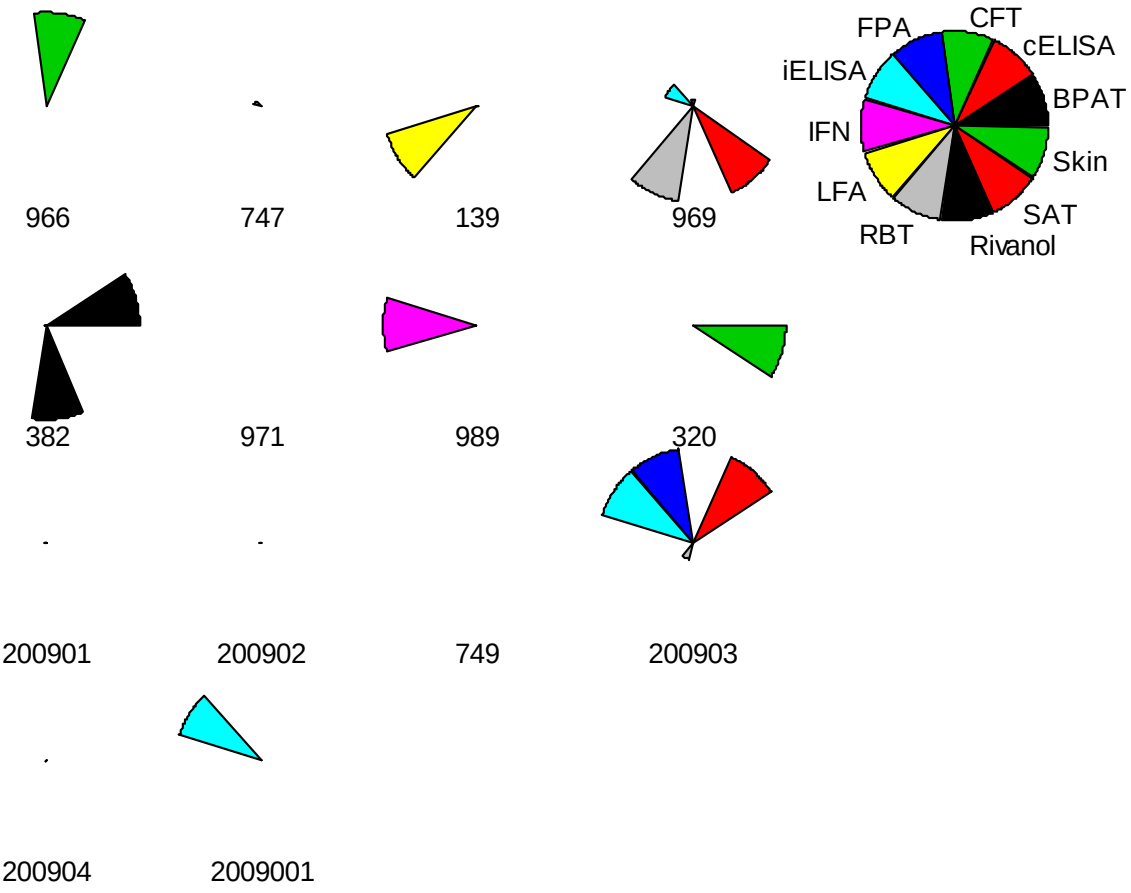

Figure 26. Impact of references on point estimates of Sp - "Leverage plot"

"Leverage plot" consisting of one pie chart for each source paper (reference ID shown below each pie chart) with sizes of slices proportional to the impact of the study on the meta-analysis summary estimate of specificity (Sp) of the respective test. Pie chart in upper right corner shows colour legend for diagnostic tests evaluated for Sp.

APPENDIX 10 – PROBABILITY MODEL

PrIntro

R-code to calculate the probability of introduction (PrIntro)

```
fSeH <- function(N,n,c=1,Se,Sp,pa) {
        # E(number of infected animals in the herd)
        EX <- max(1,N*pa)
        # E(number of test pos animals in the herd)
        EY <- EX*Se+(N-EX)*(1-Sp)
        Y  <- floor(EY)
        m  <- EY-Y              # modulus(EY,1)
        SeH<-m*phyper(c-1,Y+1,N-Y-1,n,lower.tail=F)+(1-m)*phyper(c-1,Y,N-Y,n,lower.tail=F)
        return(SeH)
        }
fSpH <- function(N,n,c=1,Se,Sp) {
        # E(number of test pos animals in the herd)
        EY <- N*(1-Sp)
        Y  <- floor(EY)
        m  <- EY-Y              # modulus(EY,1)
        SpH <-m*phyper(c-1,Y+1,N-Y-1,n)+(1-m)*phyper(c-1,Y,N-Y,n)
        return(SpH)
        }

# PrIntro
intro <- function(n,Se1,Sp1,Se2=NULL,Sp2=NULL) {
        if(is.null(Se2)){
        SePQQ <- fSeH(N=n,n=n,Se=Se1,Sp=Sp1,pa=.0001) # N=n: 1 inf animal in group
      } else {
      SePQQ  <- fSeH(N=n,n=n,Se=Se1*Se2,Sp=1-(1-Sp1)*(1-Sp2),pa=.0001)
      }
   x <- round((1-SePQQ)*(1-SePQQ)*100,dig=1)
   return(x)

# detection
detect <- function(N,n,Se1,Sp1,Se2=NULL,Sp2=NULL,pa) {
   if (is.null(Se2) | is.null(Sp2)) {
       SeCR <- Se1
       SpCR <- Sp1
       } else {
       SeCR <– 1 - (1-Se1)*(1-Se2)
       SpCR <- Sp1 * Sp2
       }
   SeCR <- fSeH(N=N,n=n,Se=SeCR,Sp=SpCR,pa=pa)
   x <- round(SeCR*100,dig=1)
   return(x)
   }
```

GLOSSARY

Feral pigs	Pigs that are raised in a free environment all of their life without any dependence on human beings (uncontrolled). Feral pigs do not exist in any part of Europe. However, to be consistent with the terminology used in EU legislation, the term of "feral pig" also covers feral wild boar.
Free ranging pigs	Owned domestic pigs allowed to range freely.
Wild boar	The wild boar and the domestic pig are members of the same species *Sus scrofa*. Wild boar are native to Europe and uncontrolled. This Opinion is concerned with uncontrolled populations of pigs in the wild, principally wild boar.

ABBREVIATIONS

AI	Artificial Insemination
BPAT	Buffered Plate Agglutination Test
BTS	Beltsville Thawing Solution
cELISA	Competitive Enzyme-Linked ImmunoSorbent Assay
CFT	Complement Fixation Test
CSF	Classical Swine Fever
EFSA	European Food Safety Authority
FPA	Fluorescent Polarisation Assay
FPSR	False Positive Serological Reactions
iELISA	Indirect Enzyme-Linked ImmunoSorbent Assay
IFN-γ	Gamma-Interferon
IFN	Gamma-Interferon Test
LFA	Lateral Flow Assay
LL	Lower Limit of 95% Credibility Interval
MA	Meta-Analysis
MCMC	Markov Chain Monte Carlo
MDMs	Microbial Diagnostic Microarrays
MLVA	Multiple Locus Variable number of tandem repeats Analysis
MS	European Union Member States
MV	Mecklenburg-West Pomeranian, Germany
NRL	National Reference Laboratory of EU
OIE	World Organization for Animal Health
OMP	Outer Membrane Proteins
ORF(s)	Open Reading Frame(s)
PATRIC	PathoSystems Resources Integration Centre
PCR	Polymerase Chain reaction
PRRS	Porcine Reproductive and Respiratory Syndrome
RBT	Rose Bengal test
RF	Risk Factor
RFLP	Restriction Fragment Length Polymorphism
Rivanol	Rivanol test
SAT	Serum Agglutination Test. TAT is a synonym.
SCC	Semen Collection Centre

Skin	Skin Test (Delayed-type Hypersensitivity test)
S-LPS	Smooth lipopolysaccharide
SNP	Single Nucleotide Polymorphism
SVD	Swine Vescicular Disease
TAT	Tube Agglutination Test. It is a synonym of SAT.
UL	Upper Limit of 95% Credibility Interval
VNTR	Variable Number of Tandem Repeats
WAHID	World Animal Health Information Database
WL	Wildlife