Part 2. Emergent diseases and biosafety

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BRUCELLOSIS — THE CURRENT SITUATION IN POLAND

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Summary. The surveys of animals for brucellosis in Poland are primarily based on serological tests. The examinations are performed by regional laboratories using RBT. In the case of positive results obtained in this test the samples are examined in SAT and CFT. The definitive confirmatory investigations are conducted by the National Reference Laboratory for Brucellosis in the Department of Microbiology of NVRI in Pulawy, which additionally uses Coombs' test, 2-Me test and ELISA. In the paper results of the examination of cattle in Poland in the years 2005–2014 are shown. Each year during examination period 130–420 cows were involved in confirmatory investigations and 6–34 animals were classified as positive for brucellosis. In bacteriological examinations of samples from seropositive cows, *B. abortus* has never been isolated. Only in 2006 *B. suis* biovar 2 has been recovered from the bovine internal organs. *B. melitensis* has never been reported and according to the Commission Decision from 2006, Poland has been regarded as 'Brucellosis officially free country'. At the moment, the aim of the ongoing testing is to maintain the *B. melitensis* free country status. Sheep and goats are tested using RBT. Samples reacting positive in the RBT are retested in the National Reference Laboratory for Brucellosis (NRL) using again RBT and CFT. Up to now all samples tested in NRL were finally regarded as negative. Ovine epididymitis (*B. ovis*) has been also reported in Poland. Diagnosis of the disease is based on the serological examination by complement fixation test (CFT) using the antigen obtained from the rough strain of *B. ovis* REO198. Similarly, examinations based on the antigen obtained from *B. canis* are conducted in dogs (rapid slide agglutination test – RSAT and slow agglutination test – SAT), but brucellosis in this species of animals has never been confirmed in Poland. Regarding the situation in porcine brucellosis, there were some outbreaks several years ago and active monitoring of pigs is performed. In general, positive results are mainly connected with false positive serological reactions (FPSR) due to *Y. enterocolitica* O:9. There is no active monitoring of brucellosis in the wildlife animals in Poland but samples are taken during the hunting season and tested for scientific purposes. When testing 4407 samples of the wild boar sera, 1077 sera reacted positive in ELISA. Also research done on hare sera several years ago indicated very similar percentage of the positives. These data show that the wildlife is a huge reservoir of the *Brucella* and it could play a role in an epidemiology of brucellosis also in domestic animals.

Keywords: *Brucella*, bovine brucellosis, porcine brucellosis, wildlife, *B. suis* biovar 2

Introduction. Brucellosis is an infectious disease, affecting many species of animals and man, caused by bacteria of the genus *Brucella*. The genus encompasses ten species: *B. abortus, B. melitensis, B. suis, B. ovis, B. canis, B. neotomae, B. cetaceae, B. pinnipediae, B. microti* and *B. inopinata* (Godfroid et al., 2005; Scholz et al., 2009; Jiménez de Bagüés et al. 2011). Brucellae are gram-negative facultative intracellular bacteria causing disease which remains a zoonosis of worldwide public health and economic importance (Godfroid et al., 2005; Franco et al., 2007). The main role play: *B. abortus*, responsible for bovine brucellosis; *B. melitensis*, the main agent of ovine and caprine brucellosis; and *B. suis*, which causes brucellosis in pigs. The testing is based almost entirely on serological assays. The most often used and important tests are Rose Bengal test (RBT), complement fixation test (CFT), ELISA, fluorescence polarization assay (FPA) and serum agglutination test (SAT). But unequivocal diagnosis of *Brucella* infection can be made only by the isolation and identification of the agent.

In Poland, bovine brucellosis has been eradicated in 1980 and since that time isolations of *B. abortus* have not been reported. In 2009 the country obtained official 'Brucellosis-free' status according to EU regulations (EC, 2009). On the other hand, *B. suis* is isolated from cattle from time to time (Szulowski et al., 2012). A huge reservoir of these bacteria constitutes a wildlife,

in particular wild boars and hares (EFSA, 2009). *B. melitensis* has never been reported and Poland has been 'Brucellosis officially free country' since 2006 (EC, 2006). What concerns brucellosis in pigs it was reported sporadically in the past but in recent years the only case concerned boars imported from one of the EU countries (Szulowski et al., 2011). In Polish conditions symptoms of *B. ovis* infections causing epididymitis, orchitis and impaired fertility in rams have been also reported. In turn, no evidence (isolation of *B. canis*) of brucellosis in dogs has been provided.

Bovine brucellosis. In Poland, bovine brucellosis (*B. abortus*) was eradicated in 1980, but due to EU regulations, the country obtained its official 'Brucellosis-free' status as late as 2009. On the other hand, wildlife constitutes a huge reservoir of *B. suis*. Surveys revealed that 12.3% of the sera from wild boars (Szulowski, Pilaszek and Iwaniak 2000) and 0.9% of the hare sera (Pilaszek, Szulowski and Iwaniak, 2000), collected from various part of the country, reacted positively in ELISA. The serology was confirmed by culture, which showed the occurrence of *B. suis* biovar 2, both in hares and wild boars (Szulowski, Pilaszek and Iwaniak 2000; Szulowski et al., 2008). The diagnosis of bovine brucellosis in Poland is primarily based on serological tests. All animals monitored for brucellosis and positive in the Rose Bengal test (RBT) undergo a further examination with the use of a serum agglutination test (SAT) and a complement fixation test (CFT) in accordance with the 'Manual of Diagnostic Tests and Vaccines for Terrestrial Animals' (OIE, 2012), and Annex C to Council Directive 64/432/EEC (EC, 2002). The National Reference Laboratory for Brucellosis (NRL) as a panel of confirmatory tests for further analysis uses also 2-mercaptoethanol test (2-ME), Coombs antiglobulin test (Coombs) and an indirect-ELISA commercial test (IDEXX). 2-Me and Coombs test are carried out according to official protocols (Królak and Stryszak, 1979; Wiśniowski, Królak and Drożdżyńska, 1978). All serum samples from those positive in RBT and SAT, which are positive in CFT and/or in one or more of additional tests (2-ME, Coombs, ELISA), in accordance with the Polish regulations, are classified as finally positive. In such cases, the animals are obligatorily slaughtered and subjected to bacteriological examination. For culture of the specimens (supramammary lymph nodes, liver, spleen, uterus) taken from the euthanized cattle the serum dextrose agar (SDA — home-made medium) is used. The plates are then incubated for 10 days at 37°C in an atmosphere with 5–10% CO_2 added. In parallel, the specimens are cultured in similar conditions in an enrichment liquid medium (serum dextrose broth — SDB, supplemented with antibiotic mixture) for up to 6 weeks with weekly subcultures on to a solid selective medium (Farrell's home-made medium). Colonies typical for *Brucella* are checked with anti-*Brucella* standard serum, examined in catalase and oxidase tests and stained by Gram's method. Further characteristics is performed by using monospecific anti-A and anti-M sera (ANSES, France) and further tests for: CO_2 requirement, production of H_2S (Hydrogen Sulfide Test Strip, Fluka) and urease, growth in the presence of thionin and basic fuchsin, and lysis by phages (Tbilisi at its routine test dilution — RTD and $10^4 \times$ RTD) (Alton et al., 1988). Additionally, molecular methods — a multiplex PCR assay (Bruce-ladder) and a multi-locus analysis of variable number tandem repeats (MLVA) are applied to confirm the identification of isolated *Brucella* strains (Lopez-Goni et al., 2008; Le Fleche et al., 2006).

From 2009, every year 1/5 of the bovine population (≥ 2 years old animals) in Poland is tested for brucellosis. Some of them react positive in a first screening test — RBT. Finally, several animals are recognised as a reacting positive for brucellosis. The next step is bacteriological examination of seroreagents. The data presented in the Table 1 show the results of serological examination of cattle in Poland from 2005 to 2014.

Table 1 – Results of serological examination of cattle in years 2005–2014

Year of testing	Number of seropositives
2005	12
2006	13*
2007	11
2008	25
2009	13
2010	34
2011	17
2012	13
2013	6
2014	19

* — isolation of *B. suis* biovar 2 from one cow

Brucellosis in sheep and goats (*Brucella melitensis* infection**).** *B. melitensis* has never been reported and according to the Commission Decision from 2006, Poland has been regarded as 'Brucellosis officially free country'. At the moment, the aim of the ongoing testing is to maintain the *B. melitensis* free country status. Sheep and goats are tested using RBT. Samples reacting positive in the RBT are retested in the National Reference Laboratory for Brucellosis (NRL) using again RBT and CFT. Up to now all samples tested in NRL were regarded as negative, it means that negative results have been obtained in CFT. If positive results in CFT are obtained, the District Veterinary Officer is asked to take the samples from the whole sheep or goat herd.

The samples are then tested again after the 30 days period from the last sampling.

Ovine epididymitis (*Brucella ovis* infection). *Brucella ovis* microorganisms cause brucellosis in sheep defined as ovine epididymitis. Disorders of reproductive tract is the main clinical sign of the disease. The disease produces inflammation of epididymis and testes in rams, infrequent abortions and poor neonatal lamb viability in ewes. Disease has been reported in many European countries, in both Americas, Africa, Australia and New Zealand. Probably disease occurs in most sheep raising countries. Ovine epididymitis has been also reported in Poland. Diagnosis of the disease is based on bacteriological or serological examination. Because of poor sensitivity, time-consuming and high costs of bacteriological methods, similarly like in diagnosis of 'smooth' *Brucella* infections serological methods are more often used. Following tests are used: complement fixation test (CFT), agar gel immunodiffusion test (AGID) and enzyme linked immunosorbent assay (ELISA). In Poland serological examination is carried out by CFT using the antigen elaborated in the National Veterinary Research Institute in Pulawy, in Department of Microbiology. The antigen has been titrated against Polish Working Standard of anti-*Brucella* ovis serum, which corresponds to International Standard of anti *Brucella ovis* serum. The antigen is in a permanent offer of the NVRI in Pulawy. At the moment, in Poland there is no ongoing national program of monitoring of *B. ovis* infection in a sheep population. Only animals devoted to reproduction, especially rams, are included in an examination scheme.

The last large scale examination has been performed in 1999. Results are shown on the Fig. 1.

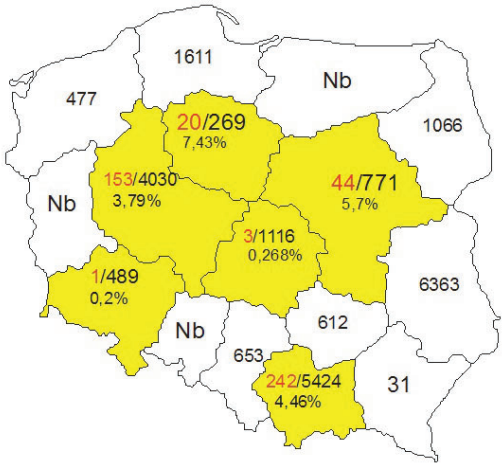

Figure 1. Distribution of the *B. ovis* seropositive sheep in voivodships in 1999

The obtained results indicate that *Brucella ovis* infections may be an important agent which affects health status and productivity of sheep flocks in Poland.

As far as *B. ovis* is not a zoonotic agent, the importance of the sheep testing has a great value, because infection in the flock leads to reduced productivity and significant economic loses in animal production.

Brucellosis in pigs. Porcine brucellosis is a zoonotic disease of widespread occurrence and global significance. However, the prevalence is low with the exception of South America and South-East Asia, where it is higher. Within the European Union (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 in Europe, and wildlife (wild boars and hares) constitutes a source of infection for pigs (Gyuranecz et al., 2011; Szulowski et al., 1999; Szulowski, Pilaszek and Iwaniak, 2000). There is a lack of systematic epidemiological data on porcine brucellosis in the member states of the EU, as there is currently no requirement for monitoring and surveillance of *B. suis* in domestic pigs and wild animals. However, in many disease-free countries statutory diagnostic testing is required, for example concerning boars in insemination stations, and is often a prerequisite for the movement of live animals. Testing is based almost entirely on serological assays, though the unequivocal diagnosis of *B. suis* infection can be made only by the isolation and identification of *Brucella*. Methods and tests used for the diagnosis of porcine brucellosis are very similar or even identical to those applied for the diagnosis of brucellosis in cattle. To date none of the serological tests has been shown to be reliable in routine diagnosis in individual pigs. The Rose Bengal test (RBT), the complement fixation test (CFT), indirect and competitive enzyme-linked immunosorbent assays (I-ELISA and C-ELISA), and the fluorescence polarization assay (FPA) are the prescribed tests for international trade purposes (OIE, 2012). In Poland the methods employed for diagnosing porcine brucellosis are RBT, I-ELISA, and additionally, to explain doubtful results, CFT, the serum agglutination test (SAT) and the 2-mercaptoethanol test (2-Me).

False positive serological reactions to brucellosis (FPSR). FPSR for brucellosis become a growing problem in international trade. The similarity of the O-antigenic side chain of *Brucella* with other microbes limits the specificity of serological diagnosis (Weiner et al., 2014). Most of the FPSR are caused by infections with *Yersinia enterocolitica* O:9, as the bacterium has identical O-antigen to that present in *Brucella* sp. (Cvetnic et al., 2003). In the last decade, *Y. enterocolitica* O:9 immensely increased the rate of FPSR (EFSA, 2007; EFSA and ECDC, 2013). The experts know the problem in their countries but there is no procedure on EU level

what to do in such case. In our opinion pigs originated from herds with the problem of FPSR for brucellosis should generally be avoided from international movement. But it is not always possible and pigs can be taken for trade from herds where the problem is not well recognized. If FPSR are found two different paths should be considered, to avoid slaughtering of animals. The first one — ignore it and introduce pigs into breeding herds. Such situation, connected with the presence of *Y. enterocolitica* O:9 infection may produce long lasting diagnostic, administrative and epidemiological problems in the future. The second path — to exclude animals from the breeding and allocate them for fattening. A clear guidelines for dealing in such cases should be created.

Brucellosis in the wildlife. The systematic brucellosis monitoring in wildlife does not exist as surveillance of the animal health status is strictly regulated for domestic animals only. But there are several publications showing the occurrence of brucellosis in wild boars in Europe. In Croatia Cvetnic et al. (2004) reported the presence of anti-*Brucella* antibodies by ELISA in 13.6% serum samples from wild boars. Garin-Bastuji et al. (2000) reported that in different regions of France positive serological reactions to brucellosis were found in wild boars in the range from 20% to 35%. In the Czech Republic the frequency of positive reactions to brucellosis was 15% (Hubálek et al., 2002) and in North-Eastern Germany 22% (Al Dahouk et al., 2005).

Last time in Poland a total of 4 407 sera of wild boars hunted in 2012, originated from territory of 11 out of 15 voivodeships from Poland were examined. Blood samples were taken from each animal from thoracic cavity, heart or pericardium into plastic tubes and allowed to clot. The sera were then separated by centrifugation and stored at – 20°C until tested. The sera have been tested by ELISA. Due to OIE Manual (2012), ELISA is one of the prescribed tests for international trade purposes in pigs. Besides, in contrast to other serological methods used in diagnosis of brucellosis, the test allows to detect anti-*Brucella* antibodies even when the quality of serum is poor, what is very common when we get material from wild animals.

Out of 4 407 examined samples, 1 077 (24.44%) reacted positively in ELISA for brucellosis. Figure 2 presents the distribution of OD values obtained in the ELISA in testing serum samples. Among sera classified as negative, the highest number of samples had OD values in the range 20–30% (N = 2 085) and 30–40% (N = 470) in relation to OD of positive control serum S+. On the other hand among sera classified as positive the largest number of samples had OD values in the range 100–150% (N = 274) and 150–200% (N = 215). The highest OD value of positive sample exceeded 800%.

Figure 3 presents the distribution of positive results of ELISA in respective voivodeships. The highest observed percentage of positive samples was observed in opolskie (39.9%), wielkopolskie (37.29%) and śląskie (34.48%) voivodeships. On the other hand the lowest ratio of positive results was observed in kujawsko-pomorskie (13.74%), łódzkie (15.47%) and warmińsko-mazurskie (18,66%).

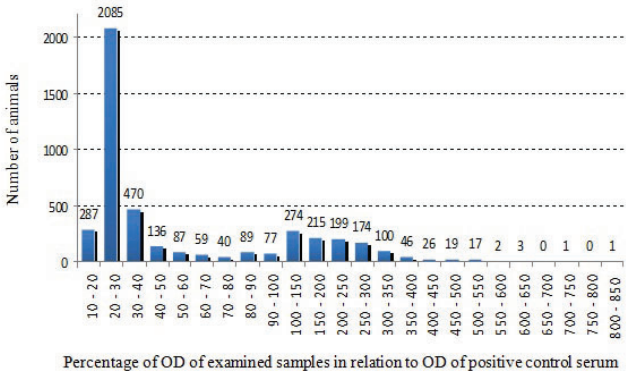

Figure 2. Distribution of OD values obtained in the ELISA in testing serum samples from wild boars

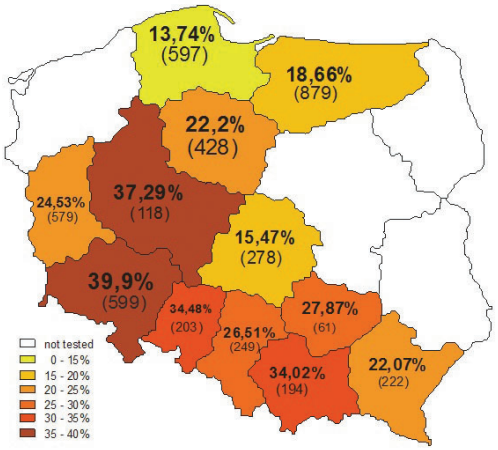

Figure 3. The results of examination of wild boars for anti-*Brucella* antibodies — voivodeships

The current results of investigations performed on much larger number of samples (N = 4 407) confirm that wild boars in Poland, similarly as in other countries of Europe, constitute a very important reservoir of *Brucella* microorganisms, undoubtedly major than hares. The prevalence, clearly higher than found several years ago, is particularly high in southwestern and lower in north and central part of the country. Bacteriological examinations performed in Poland on material from wild boars (usually lymph nodes) indicate that always the causative agent of brucellosis in wild boars and the presence of anti-*Brucella* antibodies is *B. suis* biovar 2 (Szulowski, Pilaszek and Iwaniak, 2000)

and it is typical for Europe (Kautzsch et al., 1995; EFSA, 2009). Fortunately this biovar is considered as rarely pathogenic or non-pathogenic for humans, and has only exceptionally been described as the causative agent of human brucellosis (Paton et al., 2001; Teyssou et al., 1989). But its importance stems from the fact, that *B. suis* biovar 2 can infect domestic pigs and even cattle. What concerns pigs, wild boars are potentially important source of infection especially in countries where porcine outdoor rearing systems are practiced (EFSA, 2009). It appears that only because this system is not popularized in Poland, despite so high level of *Brucella* infections in wild boars, outbreaks of brucellosis in domestic pigs are very sporadic — the last one was recorded in 1999. On the other hand, our previous investigations concerning cattle revealed that in Poland *B. suis* biovar 2 infuences the epidemiology and control of bovine brucellosis.

European brown hares along with wild boars are the natural reservoir of *B. suis* biovar 2. Small rodents and domestic animals (pigs, cattle, dogs) may be also involved in the infection chain. People can be infected while handling, skinning and eviscerating the carcases of infected hares or even by eating undercooked meat. The organism is rarely pathogenic for humans. A few years ago, in the wintertime, many hares was exported to France and to Italy. Before the shipment, the animals have been examined by palpation method. During that issue some hares showed clinical manifestation of brucellosis. The following pathological changed have been observed: nodes with purulent content, with greasy to dense consistency (more often mustard like) with yellow-green to yellow or white-creamy coloration. The nodes occurred occur in various parts of the animal body: hypodermic connective tissue, intramuscular tissue, spleen, liver, lungs and reproductive organs. Some of these changes are shown on Fig. 4–6.

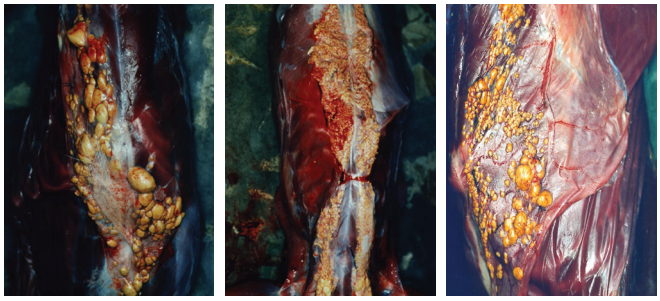

Figure 4. Purulent nodes in the subcutaneous tissue

Brucellosis in dogs. The etiological agent of brucellosis in dogs, named also contagious abortion in dogs, was first isolated by Carmichael in 1966. The causative agent is *B. canis*, similarly as *B. ovis* existing in nature in 'rough' form. The clinical signs of the disease in dogs are abortion and infertility in the female and epididymitis, orchitis, abnormal semen and testicular degeneration in the male with generalised lymph node enlargement and occasional discospondylitis and uveitis (Alton et al., 1988). In Poland the diagnosis of canine brucellosis is based on serological examinations. The antigen used in examinations has been prepared on the basis of less mucoid variant of *B. canis* strain. The methods used are the rapid slide agglutination test — RSAT, and the slow agglutination test — SAT. The examinations are not conducted in a large scale — concern primarily dogs travelling with their owners to other countries and animals from kennels. Till now *B. canis* infection has never been confirmed in Poland.

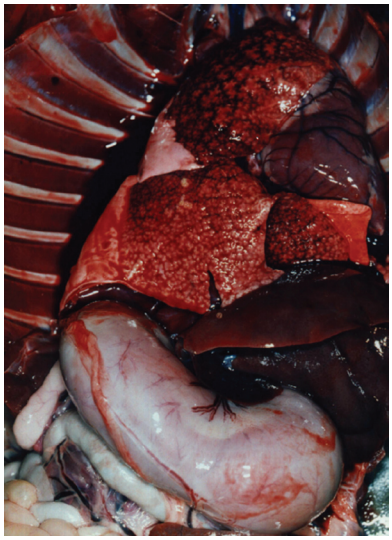

Figure 5. Caseous changes in lungs

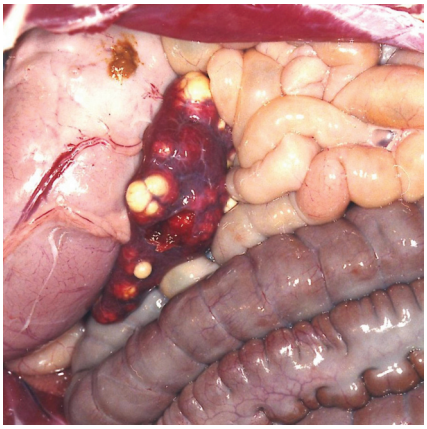

Figure 6. Enlarged spleen with purulent nodes

Conclusions. The diagnosis of brucellosis in Poland is conducted by the net of regional laboratories and the National Reference Laboratory for Brucellosis (NRL) in Department of Microbiology of the National Veterinary Research Institute in Pulawy.

The methods used are compliant with the requirements of European Community, and both serological and bacteriological methods are involved in examinations. The epidemiological situation of brucellosis in domestic animals in Poland is very good. The country has the 'brucellosis-free' status for bovine brucellosis and ovine and caprine brucellosis (*B. melitensis*). *B. melitensis* has never been confirmed in Poland while B. abortus has not been isolated from cattle since 1980. In pigs, brucellosis is recorded very rarely and, similarly as in other European countries, is caused by *B. suis* biovar 2. The reservoir of this biovar constitute wild boars (where the prevalance is relatively high) and hares. What concerns brucellosis caused by 'rough' *Brucella* species — *B. ovis* infections are recorded in sheep, whereas *B. canis* has never been confirmed in Poland.

References

Al Dahouk, S., Nockler, K., Tomaso, H., Splettstoesser, W. D., Jungersen, G., Riber, U., Petry, T., Hoffmann, D., Scholz, H. C., Hensel, A. and Neubauer, H. (2005) 'Seroprevalence of brucellosis, tularemia, and yersiniosis in wild boars (Sus scrofa) from North-Eastern Germany', *Journal of Veterinary Medicine Series B*, 52(10), pp. 444–455. doi: 10.1111/j.1439-0450.2005.00898.x.

Alton, G. G., Jones, L. M., Angus, R. D. and Verger, J. M. (1988) *Techniques for the brucellosis Laboratory*. Paris, France: Institut National de la Recherche Agronomique. ISBN 273-800-042-8.

Cvetnic, Z., Mitak, M., Ocepek, M., Lojkic, M., Terzic, S., Jemersic, L., Humski, A., Habrun, B., Sostaric, B., Brstilo, M., Krt, B. and Garin-Bastuji, B. (2003) 'Wild boars (*Sus scrofa*) as reservoirs of *Brucella suis* biovar 2 in Croatia', *Acta Veterinaria Hungarica*, 51(4), pp. 465–473. doi: 10.1556/avet.51.2003.4.4.

Cvetnic, Z., Toncic, J., Spicic, S., Lojkic, M., Terzic, S., Jemersic, L., Humski, A., Curic, S., Mitak, M., Habrun, B., Brstilo, M., Ocepek, M. and Krt, B. (2004) 'Brucellosis in wild boar (*Sus scrofa*) in the Republic of Croatia', *Veterinary Medicine (Prague) [Veterinární Medicína (Praha)]*, 49(4), pp. 115–122. Available at: http://vri.cz/docs/vetmed/49-4-115.pdf.

EC. (2002) 'Commission Regulation (EC) No 535/2002 of 21 March 2002 amending Annex C to Council Directive 64/432/EEC and amending Decision 2000/330/EC', *The Official Journal of the European Union*, L 80, pp. 22–28. Available at: http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32002R0535&qid=1442225382302&from=EN.

EC. (2006) 'Commission Decision 2006/169/EC of 21 February 2006 amending Decision 93/52/EEC as regards the declaration that Poland and certain provinces or regions of Italy are free of brucellosis (*B. melitensis*) and Decision 2003/467/EC as regards the declaration that certain provinces or regions of Italy are free of bovine tuberculosis, bovine brucellosis and enzootic bovine leucosis', *The Official Journal of the European Union*, L 57, pp. 35–40. Available at: http://eur-lex.europa.eu/legal-content/EL/TXT/PDF/?uri=CELEX:32006D0169&from=EN.

EC. (2009) 'Commission Decision 2009/600/EC of 5 August 2009 amending Decision 2003/467/EC as regards the declaration that certain Member States and regions thereof are officially free of bovine brucellosis', *The Official Journal of the European Union*, L 204, pp. 39–42. Available at: http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32009D0600&qid=1442225134857&from=EN.

EFSA. (2007) 'Scientific Opinion of the Panel on Biological Hazard (BIOHAZ) on monitoring and identification of human enteropathogenic *Yersinia* spp.', *The EFSA Journal*, 595, pp. 1–30. doi: 10.2903/j.efsa.2007.595.

EFSA. (2009) 'Scientific Opinion of the Panel on Animal Health and Welfare (AHAW) on a request from the Commission on porcine brucellosis (*Brucella suis*)', *The EFSA Journal*, 1144, pp. 1–112. doi: 10.2903/j.efsa.2009.1144.

EFSA and ECDC. (2013) 'The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2011', *The EFSA Journal*, 3129, pp. 1–250. doi: 10.2903/j.efsa.2013.3129.

Franco, M. P., Mulder, M., Gilman, R. H. and Smits, H. L. (2007) 'Human brucellosis', *The Lancet Infectious Diseases*, 7(12), pp. 775–786. doi: 10.1016/s1473-3099(07)70286-4.

Garin-Bastuji, B., Hars, J., Calvez, D., Thiebaud, M., Cau, C., Sartor, C. and Artois, M. (2000) Brucellosis in domestic pigs and wild boars due to Brucella suis biovar 2 in France. In: *Brucellosis 2000: proceedings of conference, September 7–9, Nimes, France*, pp. 44.

Godfroid, J., Cloeckaert, A., Liautard, J.-P., Kohler, S., Fretin, D., Walravens, K., Garin-Bastuji, B. and Letesson, J.-J. (2005) 'From the discovery of the Malta fever's agent to the discovery of a marine mammal reservoir, brucellosis has continuously been a re-emerging zoonosis', *Veterinary Research*, 36(3), pp. 313–326. doi: 10.1051/vetres:2005003.

Gyuranecz, M., Erdélyi, K., Makrai, L., Fodor, L., Szépe, B., Mészáros, Á. R., Dán, A., Dencsõ, L., Fassang, E. and Szeredi, L. (2011) 'Brucellosis of the European brown hare (*Lepus europaeus*)', *Journal of Comparative Pathology*, 145(1), pp. 1–5. doi: 10.1016/j.jcpa.2010.11.013.

Hubálek, Z., Treml, F., Juřicova, Z., Huňady, M., Halouzka, J., Janík, V. and Bill, D. (2002) 'Serological survey of the wild boar (*Sus scrofa*) for tularaemia and brucellosis in South Moravia, Czech Republic', *Veterinary Medicine (Prague) [Veterinární Medicína (Praha)]*, 47(2–3), pp. 60–66. Available at: http://vri.cz/docs/vetmed/47-3-60.pdf.

Jiménez de Bagüés, M. P., de Martino, A., Quintana, J. F., Alcaraz, A. and Pardo, J. (2011) 'Course of infection with the emergent pathogen *Brucella microti* in immunocompromised mice', *Infection and Immunity*, 79(10), pp. 3934–3939. doi: 10.1128/iai.05542-11.

Kautzsch, S., Seyfarth, D., Schone, R. and Stehmann, R. (1995) 'An outbreak of brucellosis in pigs and conclusions derived on the epidemiology of this animal disease', *Berliner und Münchener tierärztliche Wochenschrift*, 108(6), pp. 201–205.

Królak, M. and Stryszak, A. (1979) *Standard technique of 2-mercaptoethanol test in diagnosis of animal brucellosis [Standardowa technika odczynu z 2-merkaptoetanolem (OME) w rozpoznawaniu brucelozy zwierząt]*. Puławy: PIWet. [in Polish].

Le Flèche, P., Jacques, I., Grayon, M., Al Dahouk, S., Bouchon, P., Denoeud, F., Nöckler, K., Neubauer, H., Guilloteau, L. A. and Vergnaud, G. (2006) 'Evaluating and selection of tandem repeat loci for Brucella MLVA typing assay', [Online] *BMC Microbiology*, 6(9), pp. 1–14. doi: 10.1186/1471-2180-6-9.

Lopez-Goni, I., Garcia-Yoldi, D., Marin, C. M., de Miguel, M. J., Munoz, P. M., Blasco, J. M., Jacques, I., Grayon, M., Cloeckaert, A., Ferreira, A. C., Cardoso, R., Correa de Sa, M. I., Walravens, K., Albert, D. and Garin-Bastuji, B. (2008) 'Evaluation of a multiplex PCR assay (Bruce-ladder) for molecular typing of all *Brucella* species, including the vaccine strains', *Journal of Clinical Microbiology*, 46(10), pp. 3484–3487. doi: 10.1128/jcm.00837-08.

OIE. (2012) *Manual of diagnostic tests and vaccines for terrestrial animals (mammals, birds and bees)*. Vol. 1–2. 7th ed. Paris: OIE. ISBN 978-92-9044-878-5.

Paton, N. I., Tee, N. W.-S., Vu, C. K.-F. and Teo, T.-P. (2001) 'Visceral abscesses due to *Brucella suis* infection in a retired pig farmer', *Clinical Infectious Diseases*, 32(8), pp. e129–e130. doi: 10.1086/319748.

Pilaszek, J., Szulowski, K. and Iwaniak, W. (2000) 'Epidemiological condition of animal brucellosis in Poland' [Sytuacja epidemiologiczna brucelozy zwierząt w Polsce], *Medycyna Weterynaryjna*, 56(6), pp. 363–366. Available at: http://medycynawet.edu.pl/images/stories/pdf/digital/2000/200006363367.pdf. [in Polish].

Scholz, H. C., Nockler, K., Göllner, C., Bahn, P., Vergnaud, G., Tomaso, H., Al Dahouk, S., Kämpfer, P., Cloeckaert, A., Maquart, M., Zygmunt, M. S., Whatmore, A. M., Pfeffer, M., Huber, B., Busse, H. J. and De, B. K. (2009) '*Brucella inopinata sp. nov., isolated from a breast implant infection*', *International Journal of Systematic and Evolutionary Microbiology*, 60(4), pp. 801–808. doi: 10.1099/ijs.0.011148-0.

Szulowski, K., Iwaniak, W., Pilaszek, J., Truszczyński, M. and Chrobocińska, M. (1999) 'The ELISA for the examination of hare sera for anti-antibodies', *Comparative Immunology, Microbiology and Infectious Diseases*, 22(1), pp. 33–40. doi: 10.1016/S0147-9571(98)00019-8.

Szulowski, K., Pilaszek, J. and Iwaniak, W. (2000) 'Application of meat juice in diagnosis of brucellosis in hares and wild boars by ELISA', *The Bulletin of the Veterinary Institute in Pulawy*, 44(1), pp. 45–52.

Szulowski, K., Iwaniak, W., Pilaszek, J. and Murat, J. (2008) Wild boars and hares as reservoirs of Brucella suis biovar 2 in Poland. In: *Proceeding of the Brucellosis 2008 International Research Conference (including the 61st Brucellosis Research Conference), Septembet, 10–13, 2008, Royal Holloway College, University of London, UK*, pp. 137.

Szulowski, K., Iwaniak, W., Złotnicka, J., Weiner, M., Zaręba, Z. and Czępińska, H. (2011) 'International trade — a potential source of brucellosis in pigs', *Medycyna Weterynaryjna*, 67(1), pp. 64–66. Available at: http://medycynawet.edu.pl/images/stories/pdf/pdf2011/012011/201101064066.pdf. [in Polish].

Szulowski, K., Iwaniak, W., Weiner, M., Złotnicka, J., Szymajda, M., Zaręba, Z. and Czępińska, M. (2012) 'Diagnosis and epidemiological condition of bovine brucellosis in Poland' [Diagnostyka I sytuacja epidemiologiczna brucelozy bydła w Polsce], *Medycyna Weterynaryjna*, 68(2), pp. 110–113. Available at: http://medycynawet.edu.pl/images/stories/pdf/pdf2012/022012/201202110113.pdf. [in Polish].

Teyssou, R., Morvan, J., Leleu, J. P., Roumegou, P., Goullin, B. and Carteron, B. (1989) 'About a case of human brucellosis due to *Brucella suis* biovar 2' [A propos d'un cas de brucellose humaine a *Brucella suis* biovar 2], *Médecine et Maladies Infectieuses*, 19(3), pp. 160–161. doi: 10.1016/s0399-077x(89)80221-5. [in French].

Weiner, M., Kubajka, M., Szulowski, K., Iwaniak, W., Krajewska, M. and Lipiec, M. (2014) 'Genotypic virulence markers of *Yersinia enterocolitica* O:9 isolated from pigs and wild boars serologically positive and negative for brucellosis', *The Bulletin of the Veterinary Institute in Pulawy*, 58(4), pp. 541–545. doi: 10.2478/bvip-2014-0083.

Wiśniowski, J., Królak, M. and Drożdżyńska, M. (1978) *Standard technique of anti-globulin test in diagnosis of bovine brucellosis*. Puławy, Poland: NVRI. [in Polish].