

Isolation of *Brucella suis* biovar 2 from a wild boar in the Abruzzo Region of Italy

Fabrizio De Massis, Andrea Di Provvido, Daria Di Sabatino, Daniela Di Francesco, Katuscia Zilli, Massimo Ancora & Manuela Tittarelli

Summary

A female wild boar, aged approximately two years, was found dead by local veterinary services in Pianola di Roio in L'Aquila Province situated in the Abruzzo Region of central Italy. The carcass was submitted to the Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise 'G. Caporale' in Teramo for necropsy. *Brucella suis* biovar 2 was isolated from submandibular lymph nodes. This is the first report of isolation of *B. suis* in the Abruzzo Region. Several authors agreed in the past on the hypothesis that *B. suis* biovar 2 had been introduced into Italy through the importation of hares from European countries in which the infection is endemic in wild populations. This led the Italian authorities to reinforce existing controls for hares imported for restocking purposes. However, no provisions for brucellosis control are currently in place (or have been in place in the past) for wild boar movements either at the national or the European level. The isolation of *B. suis* biovar 2 from wild boar in two different and distant regions of Italy may suggest that this infection may have been introduced to the affected areas by wild boar rather than by imported hares. National and European rules managing wildlife brucellosis should be adapted to control the health status of farmed wild boar before movement or release, with the aim of preventing the spread of this pathogen to free territories.

Keywords

Abruzzo, *Brucella suis* biovar 2, Brucellosis, Italy, MLVA, Multiple-locus variable-number tandem-repeat analysis, Swine, Wild boar.

Introduction

Brucella suis consists of five biovars and infection in pigs is caused by biovars 1, 2 and 3. Infection of animals caused by biovars 1 and 3 differs from that caused by biovar 2 in host specificity and geographic distribution (8). Infections due to *B. suis* biovars 1 and 3 have been reported in several animal species and in humans in Europe (6, 13) but the most common *B. suis* biovar isolated in animals in Europe is biovar 2 (8). However, several human cases caused by *B. suis* biovar 2 have been reported in Europe (10, 17, 23, 28).

In domestic pigs, sporadic cases of *B. suis* infection have been reported in Austria, Denmark, France, Germany, Portugal and Spain (8). In the past five years, clinical disease has also been reported to the World Organisation for Animal Health (*Office International des Épizooties*: OIE) by Austria, Croatia, the Czech Republic, France, Germany, Montenegro, Portugal, Romania and Serbia (32). As far as wild species are concerned, isolation of *B. suis* biovar 2 has been reported in wild boar and hares, with wild boar being identified as the potential source of transmission of this biovar to outdoor or extensively reared pigs.

Serological evidence of *Brucella* spp. exposure in wild boars has been reported in many European countries such as Belgium (14), Bulgaria (21), central and south-east Europe (29), Croatia (4, 5), the Czech Republic (15), France (9), Germany (20), north-west Italy (11), Poland (27), Portugal (30), Spain (26) and Switzerland (19). However, the isolation of the agent was successful only in a few cases and scarce, if any, information exists on the distribution of this pathogen in the European wild boar population (8). In Italy, the isolation of *B. suis* biovar 2 in wild boar has been reported in a regional park in the Piedmont Region of north-western Italy (3, 11). Previously, *B. suis* biovar 2 was detected in southern Italy in a male hare (*Lepus europaeus*) that was imported from Hungary in 1995 (24). Nevertheless, serological studies which followed this report and that were conducted in Tuscany failed to reveal the exposure to *Brucella* spp. in wild boar (7) and confirmed similar results obtained in this region as those recorded in the past (12).

The aim of this paper is to describe the first case of isolation of *B. suis* biovar 2 in a wild boar in the Abruzzo Region of central Italy.

Materials and methods

Samples collected

A female wild boar aged approximately two years, was found dead by local veterinary services in Pianola di Roio in the L'Aquila Province of the Abruzzo Region in central Italy. The carcass was submitted to the *Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise 'G. Caporale'*, for necropsy. Sub-mandibular, retropharyngeal and mammary lymph nodes were collected during necropsy and submitted to isolation of *Brucella* spp. Lymph nodes collected were also subjected to standard bacteriological isolation (25) and to isolation of *Mycobacterium* spp. that was performed according to the provisions laid down in the OIE *Manual* (31).

Isolation of *Brucella* spp.

The lymph nodes were passed through a flame to sterilise the surface and were then sliced

with sterile scissors. The material obtained was then placed in a stomacher bag together with phosphate-buffered saline (PBS) and was homogenised. The suspension obtained was spread onto two modified Thayer-Martin medium plates, on two blood agar medium plates and, for enrichment purposes, on a tube of liquid Thayer-Martin medium. One Thayer-Martin medium plate and one blood agar medium plate were incubated in aerobic conditions at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$, while the remaining plates (and the liquid medium) were incubated at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ in air supplemented with 5%-10% (v/v) CO_2 . Weekly subcultures onto solid Thayer-Martin medium were performed for up to six weeks. Plates were observed after three days, and then daily, to identify the presence of bacterial colonies. Suspected colonies were subcultured and examined microscopically using the Gram stain and biochemical (urease, oxidase and catalase) and motility tests. Colonies confirmed as *Brucella* spp. were submitted to species and biovar identification (31).

Species and biovar identification

Species and biovar identification were performed by evaluating agglutination with anti-A, anti-M and anti-R monospecific sera (Veterinary Laboratories Agency, New Haw, Addlestone), the production of H_2S , CO_2 -dependence and the growth in the presence of basic fuchsin and thionin at final concentrations of $20 \mu\text{g}/\text{ml}$. Results were confirmed with AMOS-PCR (Abortus Melitensis Ovis Suis-polymerase chain reaction) and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) techniques. The AMOS-PCR amplifications were performed using the primers described in the OIE *Manual* (31). Amplification reaction mixtures were prepared in $50 \mu\text{l}$ volumes using PCR master mix 2 \times (Promega Corporation, Madison, Wisconsin). Amplifications were initiated by denaturing the sample for 5 min at 95°C , followed by 33 cycles at 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec. After the final cycle, samples were incubated for an additional 7 min at 72°C for the final extension prior to storage at 4°C . The PCR products were analysed by capillary

electrophoresis using QIAxcel Advanced System (Qiagen Srl, Milan). This was used as a 12 capillary QIAxcel DNA screening cartridge, prepared in accordance with the recommendations of the manufacturer. To ensure accurate sizing of the DNA products, a QX DNA size marker 100-3 000 bp and an alignment marker 15-3 000 bp were applied simultaneously. For RFLP, the *omp2a* and *omp31* PCR products were digested using *NcoI* and *Avall*, respectively (Promega Corporation, Madison, Wisconsin) restriction endonuclease enzymes. DNA digestions were performed in a 10 µl reaction volume containing 5 µl PCR product, 1 µl appropriate buffer, 0.4 µl endonuclease and 3.6 µl nuclease-free water. The reactions were incubated at 37°C for 2 h and ran by agarose (3%) gel electrophoresis with SYBR®-safe staining (Sigma-Aldrich Srl, Milan). The restriction patterns were displayed using an Alpha-Innotech transilluminator (Alpha-Innotech, ProteinSimple, Santa Clara, California).

Typing was aided by multiple-locus variable-number tandem-repeat analysis (MLVA) (31). Total genomic DNA was extracted using a Maxwell 16 cell DNA purification kit (Promega Corporation, Madison, Wisconsin) in accordance with the manufacturer's instructions. The resulting lysates were stored at -20°C until use. Amplification of the different variable number tandem repeats (VNTR) for MLVA-16 was performed according to the methods described previously (2, 18). PCR amplification was performed in a total volume of 25 µl containing 1 ng DNA, 1× PCR Master Mix (Promega Corporation, Madison, Wisconsin), 0.5 µM of each flanking primer and 1 M of betaine (Sigma-Aldrich Srl, Milan). Thermal cycling, conducted on a GeneAmp 9700 thermal cycler (Applied Biosystems, Carlsbad, California), was performed as follows: initial heating at 96°C for 5 m, 30 cycles denaturation at 96°C for 30 sec, annealing at 60°C for 30 sec and extension at 70°C for 60 sec. A final extension was performed at 70°C for 5 min. Regarding MLVA analysis, the QIAxcel used a standardised volume (<0.1 µl) of the PCR product for each analysis. To ensure accurate sizing of the DNA product, a QX DNA size

marker 50-800 bp (containing the molecular weight size standards of 50 bp, 100 bp, 150 bp, 200 bp, 250 bp, 300 bp, 400 bp, 500 bp, 600 bp, 700 bp and 800 bp) was used on QIAxcel runs. A reference strain of *B. melitensis* 16M was used as the known weight of the alleles providing an accurate assignment of the numbers of repetitions.

A virtual gel image and electropherogram were produced for each run, with the size of the product determined using the Biocalculator software on the same machine. The software enables the importation of data to spreadsheet software and subsequently to a conversion table in which each size is assigned to the corresponding allele.

Results

Necropsy

The animal was in good condition and presented abundant subcutaneous and perivisceral fat. The skin was blood-smearred at the snout. Massive haemothorax and haemoabdomen, with spleen rupture, was observed in natural cavities. The examination of the osteo-articular apparatus revealed the presence of a simple, complete and not open fracture of the pubic symphysis. Regional head lymph nodes showed lymphadenitis. According to the localisation and nature of lesions observed during necropsy, as well as the information provided by veterinary services, the cause of death was attributed to a car accident.

Isolation

Two weeks after the initial incubation (i.e. one week of incubation of the first subculture from liquid media), direct observation of cultures prepared from submandibular lymph nodes revealed the presence of small translucent colonies of a pale honey colour that were circular and convex in shape and were suspected to be *Brucella* colonies. Microscopic examination showed the presence of Gram-negative coccobacilli. Urease, oxidase and catalase tests gave positive results, while the motility test was negative. The other lymph nodes were negative. Lymph nodes collected

were also negative when tested using standard bacteriological isolation methods and for isolation of *Mycobacterium* spp.

Species and biovar identification

As shown in Table I, the *Brucella* isolates demonstrated agglutination only with anti-A monospecific sera. They grew in the presence of thionin but not in the presence of basic fuchsin and, like other *B. suis* biovar 2 (NCTC 10095), did not require CO₂ for primary growth and did not produce H₂S.

Results of AMOS-PCR performed on the *Brucella* strain isolated are shown in Figure 1. AMOS-PCR ruled out the possibility of the isolated strain being *B. suis* biovar 1 or 3 (Fig. 1; *B. suis* biovars 4 and 5 are not shown).

Results of the PCR-RFLP performed on the *Brucella* strain isolated are shown in Figure 2. PCR-RFLP analysis of the PCR product *omp2a* and *omp31* confirmed that the strain isolated was *B. suis* 2 and confirmed that the strain was neither *B. suis* biotype 1 nor 3 (Fig. 2).

Results of MLVA performed on the *Brucella* strain isolated are presented in Figure 3. The genetic relationships were successfully compared with those obtained by Italian and foreign strains, the data of which were published on the 2010 version of a related website (minisatellites.u-psud.fr). The allelic profile was identified as *B. suis* biovar 2 (genetic profile 2-5-8-14-5-1-5-7-4-21-9-8-12-17-

2-7). The tandem repeats (VNTR) for MLVA-16 were arranged in the following order: loci Bruce 06-08-11-12-42-43-45-55-18-19-21-04-07-09-16-30. When compared in the *Brucella* 2010 database (minisatellites.u-psud.fr) the *Brucella* strain isolated revealed a difference in marker 12, in particular when compared to genotypes 16 and 20 (MLVA8) and genotype 57 (MLVA11).

Discussion

In this study, a female wild boar that was found dead in an area of Abruzzo Region was necropsied and tissues were submitted for bacterial inspection. The wild boar was found infected with *B. suis* biovar 2. This is the first report of an isolation of *B. suis* in the Abruzzo Region of central Italy. In Italy, the isolation of this pathogen in wild boar has been reported in a regional park in the Piedmont Region of north-western Italy (11), but the study area was small and was delimited from the rest of the regional territory. Previously, *B. suis* biovar 2 was detected in southern Italy in a male hare that had been imported from Hungary in 1995 (24). Nevertheless, serological studies that followed this report in Tuscany failed to reveal the presence of *Brucella* spp. infection in wild boar (7) and confirmed similar results previously obtained in the same region (12).

Table I
Results of five classical tests, in addition to molecular tests, to identify the *Brucella* strain isolated, in comparison with other *Brucella suis* strains

Strain	Gram-stained smear	CO ₂ require-ment	H ₂ S production	Agglutination with anti-A	Agglutination with anti-M	Growth in presence of thionin	Growth in presence of basic Fuchsin
<i>Brucella suis</i> biotype 1 1330	Little coccobacilli Gram-negative	-	+	+	-	+	-
<i>Brucella suis</i> biotype 1 NCTC 10316	Little coccobacilli Gram-negative	-	+	+	-	+	-
<i>Brucella suis</i> biotype 2 NCTC 10095	Little coccobacilli Gram-negative	-	-	+	-	+	-
<i>Brucella suis</i> biotype 3 NCTC 10511	Little coccobacilli Gram-negative	-	-	+	-	+	+
Strain isolated	Little coccobacilli Gram-negative	-	-	+	-	+	-

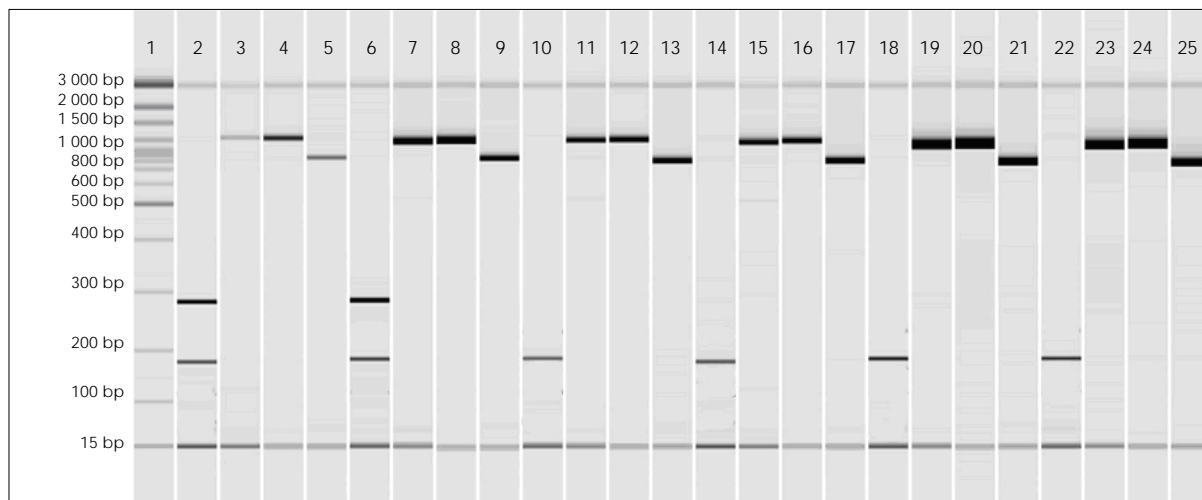


Figure 1
Results of AMOS PCR (Abortus Melitensis Ovis Suis polymerase chain reaction) on *Brucella* strain isolated compared to other *Brucella suis* strains

Lane 1: size marker

Lanes 2, 3, 4, 5: *B. suis* biotype 1 1330 (AMOS PCR, omp2a PCR, omp2b PCR, omp31 PCR, respectively)

Lanes 6, 7, 8, 9: *B. suis* biotype 1 NCTC 10316 (AMOS PCR, omp2a PCR, omp2b PCR, omp31 PCR, respectively)

Lanes 10, 11, 12, 13 and 14, 15, 16, 17: *B. suis* biotype 3 NCTC 10511 (AMOS PCR, omp2a PCR, omp2b PCR, omp31 PCR, respectively)

Lanes 18, 19, 20, 21: *B. suis* biotype 2 NCTC 10095 (AMOS PCR, omp2a PCR, omp2b PCR, omp31 PCR, respectively)

Lanes 22, 23, 24, 25: AMOS PCR, omp2a PCR, omp2b PCR, omp31 PCR of *Brucella* strain isolated, respectively

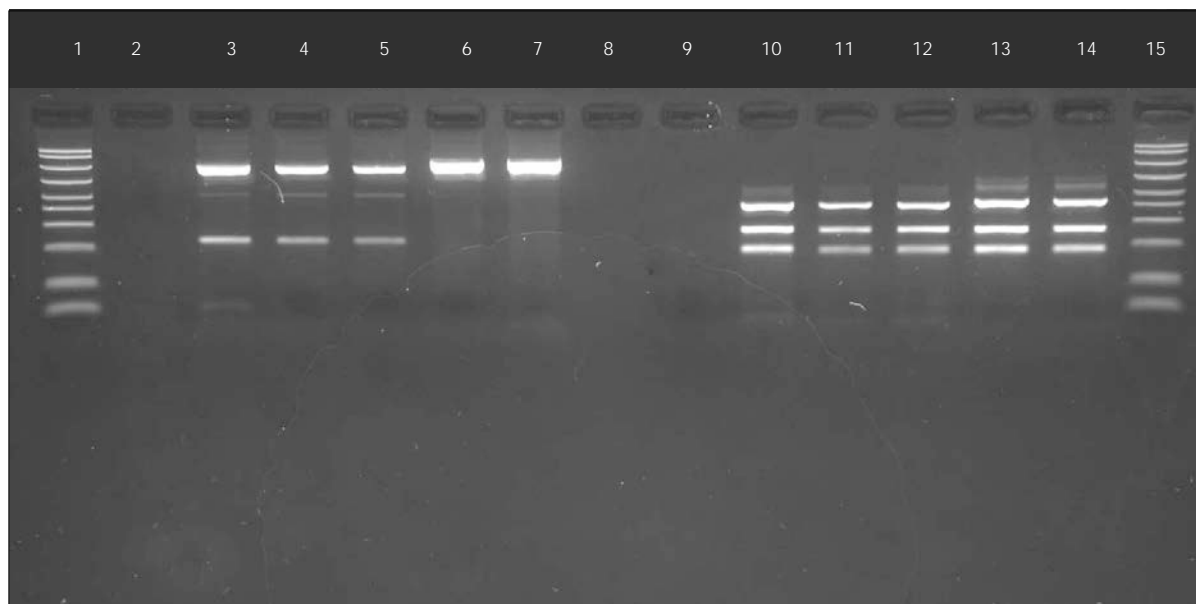


Figure 2
Results of restriction fragment length polymorphism polymerase chain reaction on *Brucella* strain isolated compared to other *Brucella suis* strains

Lanes 1 and 15: size marker (from top to bottom: 2 000 bp, 1 500 bp, 1 000 bp, 700 bp, 500 bp, 400 bp, 300 bp, 200 bp, 100 bp, 50 bp)

Lanes 3, 4, 5, 6, 7: NcoI (5' G⁺G(A/T)CC 3'/ 3'... CC(T/A)G₁ G 5') restriction pattern for omp 2a PCR product of *Brucella suis* biotype 1 1330, *B. suis* biotype 1 NCTC 10316, *B. suis* biotype 3 NCTC 10511, *B. suis* biotype 2 NCTC 10095, and *B. suis* strain isolated, respectively

Lanes 10, 11, 12, 13, 14: Ava II (5' C⁺CATG G. 3'/3' GGTAC₁C 5') restriction pattern for omp 31 PCR product of *B. suis* biotype 1 1330, *B. suis* biotype 1 NCTC 10316, *B. suis* biotype 3 NCTC 10511, *B. suis* biotype 2 NCTC 10095, and *Brucella* strain isolated, respectively

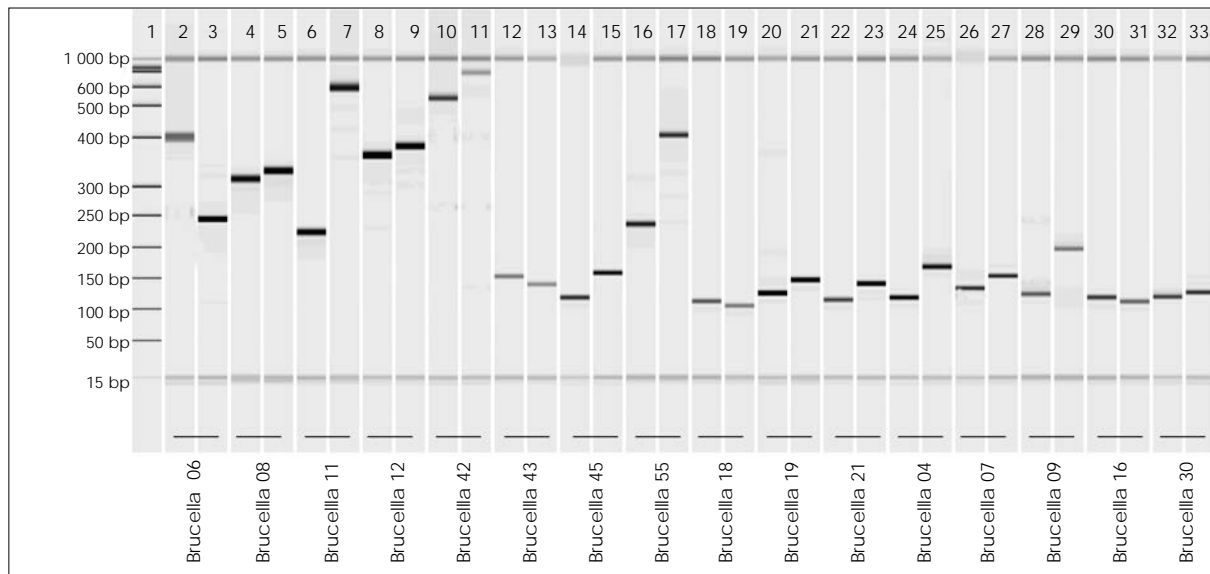


Figure 3
Amplification patterns of multiple-locus variable-number tandem-repeat analysis on *Brucella* strain isolated compared to *Brucella melitensis* biovar 1 (16M reference strain)
Even lanes: *Brucella melitensis* biovar 1 (16M reference strain)
Odd lanes: *Brucella* strain isolated
Lane 1: DNA ladder

In the past, several authors have agreed on the hypothesis that *B. suis* biovar 2 was introduced into Italy through the importation of hares from European countries in which the infection is endemic in wild populations (3, 7, 11). This hypothesis was supported by the fact that the geographic distribution of *B. suis* biovar 2 was historically recognised as confined to an area between Scandinavia and the Balkans (1, 31) and that hares from Eastern Europe, where brucellosis due to *B. suis* biovar 2 is endemic, were introduced to restock hunting areas (7). The hypothesis also considered that this species could have been a real source of *B. suis* biovar 2 infection for other animals that share the same habitat, particularly wild boars (7), as also suggested by other authors (31). This led the Italian authorities to reinforce existing controls for hares imported for restocking purposes (16).

Wild boar were also imported from Eastern Europe during the last few decades (7). In Italy, wild boar restocking for hunting purposes commenced in the 1950s and was initially organised with animals imported from abroad. The releases continued thereafter, mainly with captive animals farmed in Italy. However, still today, several provincial

administrations, especially those in the south of the country, buy farmed wild boar directly for restocking or they authorise other bodies (hunting companies, wildlife management companies, etc.) to regularly release farmed wild boars. Some regions have banned wild boar farming for restocking purposes and have also explicitly prohibited the release on their territory (22).

Our report provides the first confirmation of the presence of *B. suis* biovar 2 in a previously free area and can probably be explained by imports of wild boars. A comprehensive study is required on the prevalence of this organism in wild boar and an evaluation of the risks to indoor livestock production.

To confirm and identify species and biovar level, phenotypic characterisation or classical bio-typing methods remain the definitive diagnosis and 'gold standard'. AMOS-PCR and RFLP-PCR are highly reliable, accurate and reproducible tests; other advantages are speed, minimal sample preparation and reduced risk of exposure. MLVA is not usually recommended for typing isolates at species level. However, as shown in this study, this technique may provide additional molecular epidemiological information on the specific

profile of this isolate and this may be of value in future encounters with *B. suis* biovar 2 isolates, thus enlarging the potential of MLVA as an epidemiological tool for brucellosis diagnosis.

Conclusions

No provisions for brucellosis control are currently in place (or have been implemented in the past) for wild boar movements both at national and European levels. Only controls of hares imported for restocking from abroad are in place at the national level. The isolation of *B. suis* biovar 2 from wild boar is reported here and reference is made to a similar report in a different and distant region of Italy (11) that

may suggest the possibility that this infection may have been introduced in the affected areas by wild boar rather than by imported hares. National and European rules that govern wildlife brucellosis should be implemented to monitor the health status of farmed wild boar prior to authorisations for movement or release. This would help prevent the spread of this pathogen to disease-free areas.

References

1. Alton G.G. 1990. *Brucella suis*. In Animal brucellosis (K. Nielsen & J.R. Duncan, eds). CRC Press, Boston, 453 pp.
2. Al Dahouk S., Le Flèche P., Nöckler K., Jacques I., Grayon M., Scholz H. C., Tomaso H., Vergnaud G. & Neubauer H. 2007. Evaluation of *Brucella* MLVA typing for human brucellosis. *J Microbiol Methods*, **69**, 137-145.
3. Bergagna S., Zoppi S., Ferroglio E., Gobetto M., Dondo A., Di Giannatale E., Gennero M.S. & Grattarola C. 2009. Epidemiologic survey for *Brucella suis* biovar 2 in a wild boar (*Sus scrofa*) population in northwest Italy. *J Wildl Dis*, **45**, 1178-1181.
4. Cvetnić Z., Mitak M., Ocepek M., Lojkić M., Terzić S., Jemersić L., Humski A., Habrun B., Sostarić B., Brstilo M., Krt B. & Garin-Bastuji B. 2003. Wild boars (*Sus scrofa*) as reservoirs of *Brucella suis* biovar 2 in Croatia. *Acta Vet Hung*, **51**, 465-473.
5. Cvetnić Z., Tončić J., Spicić S., Lojkić M., Terzić S., Jemersić L., Humski A., Curić S., Mitak M., Habrun B., Brstilo M., Ocepek M. & Krt B. 2004. Brucellosis in wild boar (*Sus scrofa*) in the Republic of Croatia. *Vet Med (Praha)*, **49**, 115-122.
6. Cvetnić Z., Spicić S., Curić S., Jukić B., Lojkić M., Albert D., Thiébaud M. & Garin-Bastuji B. 2005. Isolation of *Brucella suis* biovar 3 from horses in Croatia. *Vet Rec*, **156**, 584-585.
7. Ebani V.V., Cerri D., Poli A. & Andreani E. 2003. Prevalence of *Leptospira* and *Brucella* antibodies in wild boars (*Sus scrofa*) in Tuscany, Italy. *J Wildl Dis*, **39**, 718-722.
8. European Food Safety Authority (EFSA) 2009. Scientific Opinion of the Panel on Animal Health and Welfare (AHAW) on a request from the Commission on porcine brucellosis (*Brucella suis*). *EFSA J*, **1144**, 1-112.
9. Garin-Bastuji B., Hars J., Thiébaud M. & Artois M. 2000. Brucellosis of domestic pigs. Reemergence of *Brucella suis* biovar 2 in France. *Epidémiol Santé Anim*, **38**, 1-5.
10. Garin-Bastuji B., Vaillant V., Albert D., Tourrand B., Danjean M.P., Lagier A., Rispal P., Benquet B., Maurin M., De Valk H & Mailles A. 2006. Is brucellosis due the biovar 2 of *Brucella suis* an emerging zoonosis in France? Two case reports in wild boar and hare hunters. In Proc. 1st International meeting on treatment of human brucellosis, 7-10 November, Ioannina. International Society of Chemotherapy Disease Management, Ioannina (document available on CD).
11. Gennero M.S., Grattarola C., Zoppi S., Di Giannatale E. & Dondo A. 2004. Brucellosis in wild boars in Piedmont Region. *Epidémiol Santé Anim*, **45**, 77-79.
12. Giovannini A., Cancellotti F. M., Turilli C. & Randi E. 1988. Serological investigations for some bacterial and viral pathogens in fallow deer (*Cervus dama*) and wild boar (*Sus scrofa*) of the San Rossore Preserve, Tuscany, Italy. *J Wildl Dis*, **24**, 127-132.
13. Godfroid J. 2002. Brucellosis in wildlife. *Rev Sci Tech*, **21**, 277-286.

14. Godfroid J., Michel P., Uytterhaegen L., De Smedt C., Rasseneur F., Boelaert F., Saegerman C. & Patigny X. 1994. Brucellose enzootique à *Brucella suis* biotype 2 chez le sanglier (*Sus scrofa*) en Belgique. *Ann Med Vet*, **138**, 263-268.
15. Hubálek Z., Tremel F., Juřicová Z., Huřády M., Halouzka J., Janík V. & Bill D. 2002. Serological survey of the wild boar (*Sus scrofa*) for tularaemia and brucellosis in South Moravia, Czech Republic. *Vet Med (Praha)*, **47**, 60-66.
16. Italian Ministry of Health 2000. Norme sanitarie per l'importazione di lepri destinate al ripopolamento. Decreto 7 dicembre 2000. *Gazz Uff*, **38**, 15 February 2001.
17. Lagier A., Brown S., Soualah A., Julier I., Tourrand B., Albert D., Reyes J. & Garin-Bastuji B. 2005. Brucellose aiguë à *Brucella suis* biovar 2 chez un chasseur de sanglier. *Med Mal Infect*, **35**, 185.
18. Le Flèche P., Jacques I., Grayon M., Al Dahouk S., Bouchon P., Denoëud F., Nöckler K., Neubauer H., Guilloteau L.A. & Vergnaud G. 2006. Evaluation and selection of tandem repeat loci for a *Brucella* MLVA typing assay. *BMC Microbiol*, **6**, 9.
19. Leuenberger R., Boujon P., Thür B., Miserez R., Garin-Bastuji B., Rüfenacht J. & Stärk K.D.C. 2007. Prevalence of classical swine fever, Aujeszky's disease and brucellosis in a population of wild boar in Switzerland. *Vet Rec*, **160**, 362-368.
20. Melzer F., Lohse R., Nieper H., Liebert M. & Sachse K. 2006. A serological study on brucellosis in wild boars in Germany. *Eur J Wildl Res*, **53**, 153-157.
21. Mineva I., Likov B., Minev M., Makedonski I., Kaloianov Z. & Sandev R. 1991. Intensity and dynamics of epizootological process caused by *Brucella suis* in Shumen region for the period 1978-1990. In Bulletin of the Scientific Conference of Regional Veterinary Institute, Stara Zagora, 24 October 1991.
22. Monaco A., Carnevali L. & Toso S. 2010. Linee guida per la gestione del Cinghiale (*Sus scrofa*) nelle aree protette, 2° edizione. Quad Cons Natura, 34, Italian Ministry of the Environment, ISPRA, Rome, 121 pp.
23. Paton N.I., Tee N.W.S. Vu C.K.F. & Teo T.P. 2001. Visceral abscesses due to *Brucella suis* infection in a retired pig farmer. *Clin Infect Dis*, **32**, 129-130.
24. Quaranta V., Farina R., Poli A., Cerri D. & Palazzo L. 1995. Sulla presenza di *Brucella suis* biovar 2 nella lepre in Italia. *Selezione Vet*, **36**, 953-958.
25. Quinn P.J., Carter M.E., Markey B.K. & Carter G.R. 1994. Clinical veterinary microbiology. Wolfe Publishing, New York, 648 pp.
26. Ruiz-Fons F., Vicente J., Vidal D., Höfle U., Villanúa D., Gauss C., Segalés J., Almería S., Montoro V.L. & Gortázar C. 2006. Seroprevalence of six reproductive pathogens in European wild boar (*Sus scrofa*) from Spain: the effect on wild boar female reproductive performance. *Theriogenology*, **65**, 731-743.
27. Szulowski K., Iwaniak W., Pilaszek J. & Murat J. 2008. Wild boars and hares as reservoirs of *Brucella suis* biovar 2 in Poland. In Proc. Brucellosis 2008 International Research Conference (including the 61st Brucellosis Research Conference), 10-13 September, London. Veterinary Laboratories Agencies, London, 224 pp.
28. Teyssou R., Morvan J., Leleu J.P., Roumegou P., Goullin B. & Carteron B. 1989. About a case of human brucellosis due to *Brucella suis* biovar 2. *Med Mal Infect*, **19**, 160-161.
29. Taleski V., Zerva L., Kantardjiev T., Cvetnić Z., Erski-Biljic M., Nikolovski B., Bosnjakovski J., Katalinić-Janković V., Panteliadou A., Stojkoski S. & Kirandziski T. 2002. An overview of the epidemiology and epizootology of brucellosis in selected countries of central and southeast Europe. *Vet Microbiol*, **90**, 147-155.
30. Vaz Y., Rodeia S. & Corrêa de Sá M.I. 2004. Available data on *Brucella suis* in Portugal. Oral communication at COST 845 Brucellosis in animals and man *B. suis* meeting, Laboratorio Nacional de Investigaçao Veterinaria (LNIV), 6-7 May, Lisbon. LNIV, Lisbon (document available on CD).
31. World Organisation for Animal Health (Office International des Épizooties: OIE) 2011. Manual of diagnostic tests and vaccines for terrestrial animals 2011. Chapter 2.4.3. Bovine brucellosis. OIE, Paris, 1 343 pp. (www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.04.03_bovine_bruce.pdf accessed on 1 August 2012).
32. World Organisation for Animal Health (Office International des Épizooties: OIE) 2011. World Animal Health Information Database (WAHID) Interface. OIE, Paris (web.oie.int/wahis/public.php?page=disease_timelines accessed on 1 August 2012).