MYCOBACTERIOSIS IN WILD BOAR: RESULTS OF 2000-2006 ACTIVITY IN NORTH-WESTERN ITALY *

Alessandro Dondo [1], Simona Zoppi [1], Francesca Rossi [1], Laura Chiavacci [1], Antonio Barbaro [2], Annalisa Garrone [1], Alessandro Benedetto [1] and Maria Goria [1]

***SUMMARY**: This work reports the results of our investigation on the presence of* Mycobacteria *in wild boar shot down in Piedmont, Liguria and the Aosta Valley between 2000 and 2006.*

1254 submandibular lymph nodes were collected to detect Mycobacterium *spp. ; suspected colonies were identified and typed by molecular methods (Multiplex PCR, RD Region analysis, Variable Number Tandem Repeats, Spoligotyping).*

Most strains of M. bovis *were isolated in the Liguria region, while in the Piedmont region* M. bovis *was found to be sporadic. Wild boars may also be infected by other* Mycobacteria*, e.g. M.* microti*, presumably by eating infected dead small rodents. In view of their rooting behaviour, wild boars are exposed to infection by various species of* Mycobacteria *other than M. tb complex.*

Keywords: *Mycobacterium* spp.*,* North Western Italy, wild boar.

***RESUME** : Ce travail fournit les résultats relatifs à la recherche des mycobactéries chez les sangliers tués à la chasse au Piedmont, en Ligurie et en Vallée d'Aoste entre 2000 et 2006.*

Mille deux cent cinquante quatre ganglions sousmandibulaires ont été récoltés pour rechercher Mycobacterium *spp.et soumis à des tests moléculaires divers (Multiplex PCR, RD Region analysis, Variable Number Tandem Repeats, Spoligotyping).*

La plupart des souches ont été isolées en Ligurie ; au Piedmont, au contraire, l'isolement a été sporadique.

Mais d'autres mycobactéries, par exemple M. microti*, peuvent infecter le sanglier après l'ingestion de petits rongeurs infectés.*

Le fouissement du sol augmente le risque, pour le sanglier, d'être infecté par plusieurs espèces de mycobactéries autres que le complexe M. tb.

Mots-clés : *Mycobacterium* spp.*,* Nord Ouest Italie, sanglier.

* Texte de la communication orale présentée lors de la Journée AEEMA, 1er juin 2007

1 Istituto Zooprofilattico Sperimentale di Piemonte, Liguria e Valle d'Aosta, Via Bologna 148, 10154 Torino, Italy; alessandro.dondo@izsto.it

2 Osservatorio Epidemiologico Regione Piemonte, Italy

I - INTRODUCTION

Wild animals can be a reservoir for *Mycobacteria.* Indeed they may act as vectors of *M. bovis* for cattle. They may also give an indication of the *mycobacterial* contamination of the environment. The presence of bovine tuberculosis in a wild animal population is generally not easy to control and it raises problems of public health and wildlife management. It may interfere with the eradication programmes. Wild boars may be carriers of infection with various species of *Mycobacteria* other than *M. tb* complex group. As long as their role is not clarified, it seems more appropriate to refer to mycobacteriosis to define these diseases in wild boars in North-Western Italy,

M. bovis infection in wild boars was reported in several European countries [Hermoso de Mendoza *et al.*, 2006; Karina Acevedo-Whitehouse *et al.*, 2005, Machakowa *et al.*, 2003, Vicente *et al.*, 2006], Italy included [Bollo *et al.*, 2002; Bollo *et al.*, 2000; Boniotti *et al.*, 2005; Dini *et al.*, 2003; Ferroglio *et al.*, 1996]: The first evidence was reported in Germany in 1934 [Kindinger *et al.*, 1934]. Despite the data of the literature, it is convenient to distinguish sporadic cases from endemic infections in this species. Most reported cases [Machakowa *et al.*, 2003] refer to tuberculosis caused by *M. bovis* in pasture areas shared with cattle herds. The incidence of *M. bovis* infection in wild boars is directly related to the prevalence and incidence of the disease in cattle in the same area.

In certain areas of Northwestern Italy, it was shown that the prevalence of *M. bovis* in wild boars is related to the prevalence in cattle since they share the same habitat and pasture areas. In some cases, a sylvestrian cycle appeared likely [Bollo *et al.*, 2002; Bollo *et al.*, 2000; Dini *et al.*, 2003].

II - MATERIALS AND METHODS

1. BACTERIOLOGICAL EXAMINATION

After removal of connective and fat tissue, lymph nodes samples were homogenized and decontaminated in two different ways (hexadeciylpyridinium chloride 1.5% and Sodium Hydroxide 2%). Both were then inoculated onto three different types of solid media: Lowestein Jensen, Stonebrink and Lowestein Jensen w/o glycerin. The solid media were incubated for 10 days at 37°C under 5% CO_2 and then for 80 days at 37°C. Suspected colonies were collected for identification and typing by molecular methods.

2. MOLECULAR IDENTIFICATION AND TYPING OF STRAINS

DNA from isolated strains was extracted by heat shock at 99°C for 20 min in water; Identification of the isolates was performed by using a multiplex PCR, an in house variation of protocols (data not shown) described by Kulski [Kulski *et al*., 1995] and by Sinclair [Sinclair *et al.*, 1995].

This Multiplex PCR is based on simultaneous detection of different molecular target: RNAr16S sequences, insertion element IS986 and mpt40 gene. Different *Mycobacteria* species are detected by the electrophoretic profile of the PCR product. The strains classified as *M. tb* Complex cluster, including other members of *Mycobacteria* (*M. tuberculosis, M. africanum, M. microti, M. bovis, M. bovis* BCG), were then typed using Spoligotyping, usually applied for molecular characterisation, but very useful to differentiate *M. bovis* from *M. tuberculosis.* Spoligotyping was performed as described by Kamerbeek [Kamerbeek *et al.*, 1997]. This technique is based on *in vitro* amplification of the DNA of the highly polymorphic DR genomic locus present in the *M. tb* Complex chromosome. This locus contains multiple, well preserved 36-bp direct repeats sequences (DRs) interspersed with non-repetitive spacer sequences. Strains vary in the number of DRs and in the presence or absence of these spacer sequences. They could be characterised by specific absence/presence by

a PCR based reverse line blot method covalently (Isogen, Netherland).

M. bovis strains isolated were further characterized by VNTR typing (ETR A, B, C, D, E) according to Frothingham [Frothingham *et al.*, 1998].

The strains unidentified by Spoligotyping were processed by a PCR-based *M. tb* Complex typing method that makes use of *M. tb* Complex chromosomal region of difference deletion *loci*. According to Huard [Huard *et al.*, 2003] three primer pairs (which amplify within the *loci* Rv1510, Rv3120 and IS1561) were run in separate but simultaneous reactions. The pattern of PCR products from all the reactions allowed immediate identification either as *M. tb* Complex or as *M. microti*.

3. EPIDEMIOLOGICAL CORRELATION

The GIS program was applied to show in parallel the distribution on regional territories of hunting zones and that of cattle pasture areas to compare strain features.

III - RESULTS

252 out of 1254 samples examined led to the isolation of *Mycobacteria* strains: *M. bovis* (n= 38), *M. microti* (n=89), *M. avium* (n=19), *Mycobacteria spp* (n=110). Six strains could not be positively classified as *Mycobacterium Tb complex* other than *M. bovis*, *M. tuberculosis* or *M. microti*.

Spoligotype and VNTR profile of 38 *M. bovis* strains were subsequently compared to those isolated in cattle coming from the same areas during the study period.

The GIS program showed the simultaneous presence of homologous strains in wild boar and cattle populations in the same areas in the same period (figures 1, 2 and 3).

IV - DISCUSSION

Wild boars are more exposed than other animal species to contacts with *Mycobacteria* in the environment, presumably because of their behaviour and eating habits. They may eat dead small rodents while rooting. However, this contamination does not seem to cause a clinical disease, presumably because of the wild boars' genetic resistance to the infection [Karina Acevedo-Whitehouse *et al*., 2005]. At the most it may produce lesions detected by pathological examination: granulomatous reactions limited to the submandibular lymph nodes.

Our findings on *M.bovis* strains isolated and typed by molecular tests revealed that, in this context, spoligotype BCG-*like* strains are more widespread than others. VNTR typing can be used to further differentiate them.

Thanks to Spoligotype and VNTR profiles, wild boar strains were compared to bovine strains isolated in the same area. Through a spatial analysis, we observed in some cases a similarity between strains isolated from cattle and strains isolated from wild boars in the same areas: particularly in the provinces of Cuneo, Imperia and Savona.

Our data suggest that it is unlikely that wild boars act as a reservoir for bovine tuberculosis. On the other hand, the infection in wild boars may be a useful marker of the presence of *M.bovis* in the environment and of exposure of farm animals.

The eating behaviour of wild boars (rooting) and the site of the lesions (mostly in the submandibular lymph nodes) suggest that the contamination of wild boars occurs mostly by the oral route rather than by contact with infected animals (transversal dissemination).

In conclusion, on the basis of the results obtained in this study (finding of small lesions in head lymph nodes; sensitivity of isolation technique lower than that of molecular detection in the same tissue samples), we suggest that infection in wild boars provides a sensitive marker of *Mycobacteria* environmental contamination, but that wild boars hardly represent a reservoir for disease spreading.

Figure 1

Geographical limits of areas under hunting jurisdiction
(Ambito Territoriale di Caccia - ATC and Comprensorio Alpino - CA)

Location of boar-hunting areas (in grey) and cattle farms (in black) considered in this study

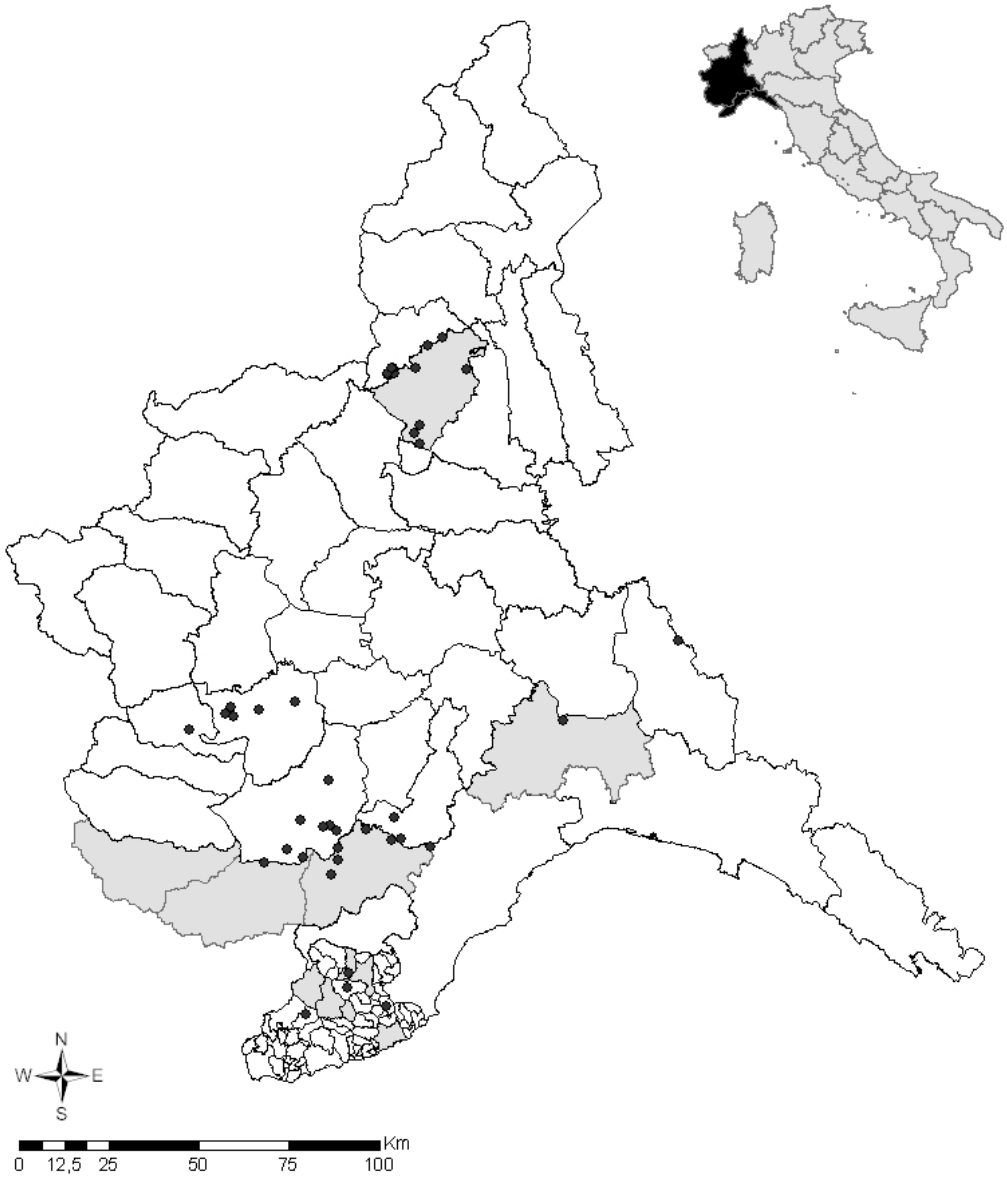
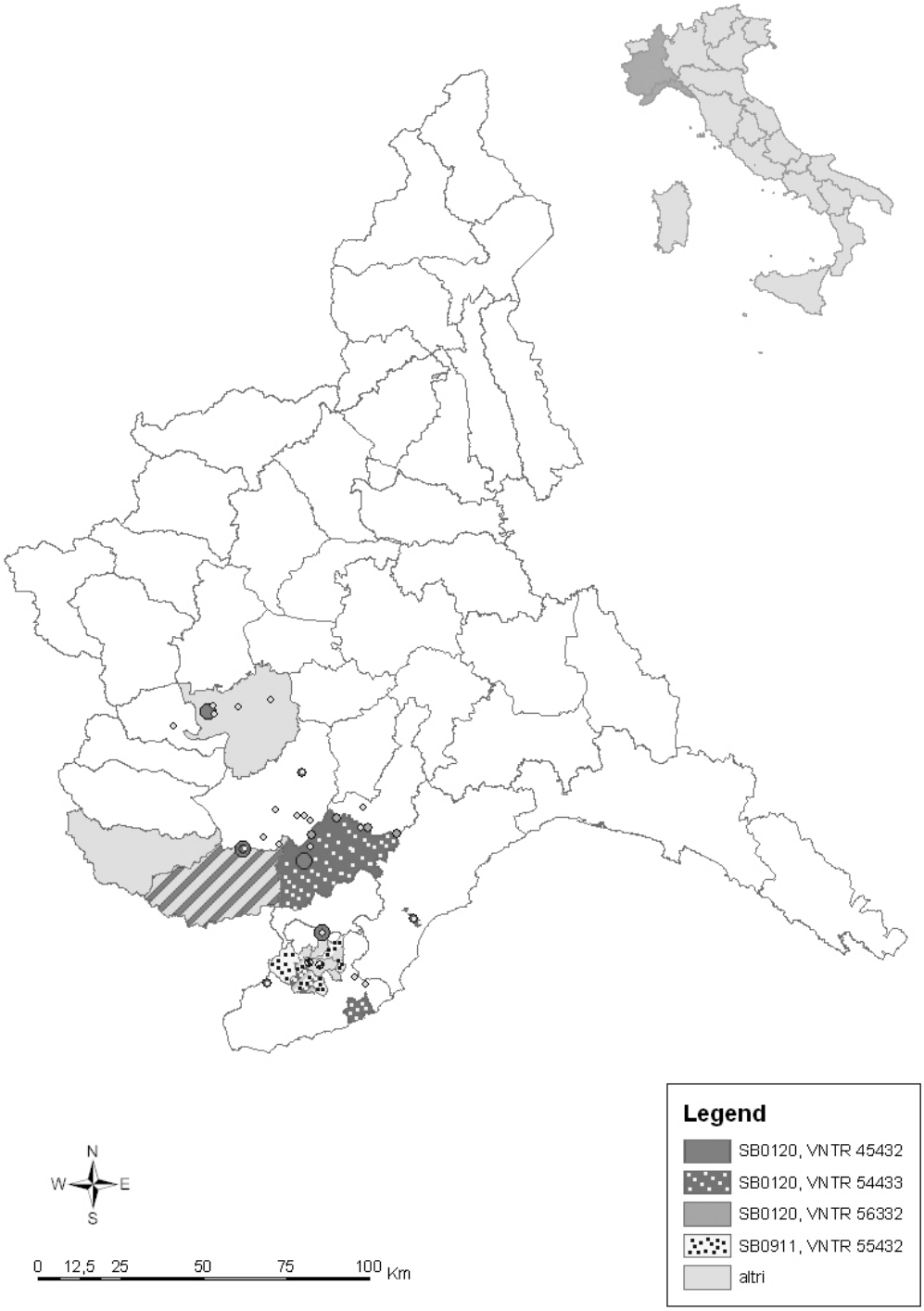


Figure 3
Representation of molecular profile homology between cattle- and wild boar *M. bovis* strains. The figure shows that *M. bovis* strain SB0911 VNTR 55432 is only present in cattle and wild boar in the Liguria region. *M. bovis* SB0120 is the most frequent strain profile in our territory.



BIBLIOGRAPHY

AA.VV. - Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 2004 (O.I.E.).

Bollo E., Ferroglio E. - La tubercolosi negli animali selvatici. *Obiettivi e Documenti Veterinari*, 2002, **23**(5), 57-67.

Bollo E., Ferroglio E., Dini V., Mignone W., Biollatti B., Rossi L. - Detection of *Mycobacterium tuberculosis* Complex in Lymph Nodes of Wild Boar (*Sus scrofa*) by a Target-Amplified Test System. *Journal of Veterinary Medicine*, 2000 Series B 47 (5), 337-342.

Boniotti M.B., Goria M., Garrone A., Benedetto A., Dondo A., Zoppi S., Mondo A., Bonora S., Zanardi G., Avisani D., Zanoni M., Tagliabue S. and Pacciarini M.L. – Molecular typing of *M. bovis* strains from Northern Italy –4th International Conference on *Mycobacterium bovis* – Dublin, Ireland 22/26 August 2005.

Brosch R. et al. -. A new evolutionary scenario for the *Mycobacterium tuberculosis* Complex. *PNAS* 2002, **99**(6), 3684-3689.

Dini V., Ferroglio E., Serraino A., Mignone W., Sanguinetti V., Bollo E., Rossi L. - Epidemiologia delle micobatteriosi nel cinghiale in Liguria. *J. Mt. Ecol*., 2003, **7** (Suppl.), 145 – 153.

Dondo A., Zoppi S., Angelillo M., Buonincontro G., Garrone A., Squadrone S., Benedetto A., Goria M. - Diagnosi post mortem di tubercolosi nei bovini: esiti e considerazioni sul protocollo diagnostico applicato nel periodo 2001-2004 in Piemonte. – *Il Progresso Veterinario*, 2005, n. **8**, 351-357.

Dondo A., Zoppi S., Goria M., Garrone A., Chiavacci L., Travaglio S., Caroggio P., Mignone W. - *M. bovis* infection in wild boar and cattle: an overview of data sampled in North-Western Italy during the years 1998-2004 - 4th International Conference on Mycobacterium bovis – Dublin, Ireland 22/26 August 2005.

Ferretti B. *et al*. - Identification and Spoligotyping of M. bovis strains isolated in Italy from different animal species and man. *Rev. Portuguesa Pneumol.*, 1998, **4**(3), 360.

Ferroglio E., Rossi L. - Brevi note informative sulla tubercolosi nel cinghiale ed in altri mammiferi selvatici. *Med. Vet. Prev*., 1996, **12**, 8-9.

Frothingham, R., Meeker-O'Connell, W.A. - Genetic diversity in the Mycobacterium tuberculosis Complex based on variable numbers of tandem DNA repeats. *Microbiology*, 1998, **144**, 1189-1196.

Hermoso de Mendoza J., Parra A., Tato A., Alonso J.M., Rey J.M., Pena J., Garcia-Sanchez A., Larrasa J., Teixido J., Manzano G., Cerrato R., Pereira G., Fernandez-Llario P., Hermoso de Mendoza M. - Bovine tuberculosis in wild boar (Sus scrofa), red deer (Cervus elaphus) and cattle (Bos taurus) in a Mediterranean ecosystem (1992-2004). *Prev. Vet. Med.*, 2006, 239-47.

Huard R.C. *et al*. - PCR-Based method to differentiate the subspecies of the Mycobacterium tuberculosis Complex on the basis of genomic deletions. *J. Clin. Microbiol*., 2003, **41** (4).

Kamerbeek J. *et al*. - Simultaneous detection and strain differentiation of Mycobacterium tuberculosis for diagnosis and epidemiology. *J. Clin. Microbiol*., 1997, **35**, 907-914.

Karina Acevedo-Whitehouse K., Vicente J., Gortazar C., Höfle U.G., Fernández-De-Mera I.G., Amos W. - Genetic resistance to bovine tuberculosis in the Iberian wild boar. *Molecular Ecology*, 2005, **14**(10), 3209.

Kindinger H. - Tuberculose beim wild in freier wildbahn. Vet. Med.Dissertation Giessen, 50 pp.

Kulski J.K. *et al*. - Use of a Multiplex PCR to detect and identify M. avium and M. intracellulare in blood culture fluid of AIDS patients. *J. Clin. Microbiol*., 1934, **33**(8), 668-674.

Serraino A., Marchetti G., Sanguinetti V., Rossi M.C., Zanoni R.G., Catozzi L., Bandiera A., Dini W., Mignone W., Pranzetti F., Gori A. - Monitoring of transmission of tuberculosis between wild boars and cattle: genotypical analysis of strains by molecular epidemiology techniques. *J. Clin. Microbiol*., 1999, **37**, 2766-71.

Machakowa M., Matlova L., Lamka J., Smolik J., Melicharek I., Hanzlikova M., Docekal J., Cvetnik Z., Nagy G., Lipiec M., Ocepek M., Pavlik I. - Wild boar (*Sus scro*fa) as a possible vector of mycobacterial infections: review of literature and critical analysis of data from Central Europe between 1983 and 2001. *Vet. Med. - Czech*, 2003, **48** (3), 51-65.

Sinclair K. *et al*. - A Multiplex PCR for distinguishing Mycobacterium tuberculosis Complex. *Mol.Cell. Probes*, 1995, **9**, 291-295.

Vicente J., Höfle U., Garrido J.M., Fernández-De-Mera I.G., Juste R, Barral M., Gortazar C. - Wild boar and red deer display high prevalences of tuberculosis-like lesions in Spain. *Vet. Res*., 2006, **37**, 107-119.