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Polymerase chain reaction method for leptospirosis, analysis on samples from an autochthon swine population in Sicily, Italy

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SUMMARY

We set a method targeting 16 rRNA gene consisting in a single polymerase chain reaction of 40 cycles which is specific for pathogenic leptospira. Negative polymerase chain reaction results were observed with nonpathogenic *Leptospira* (serovar patoc¹) and other bacteria species. By this method a survey on a population of autochthon swine herds had been conducted in Sicily particularly on kidney samples of slaughtered animals and on urine samples from live animals. The analysis showed that a prevalence of leptospira up to 40 % can be observed on these animals. Results on other bovine and ovine herds from the same province in Sicily showed a lower prevalence.

Key words: Animal Leptospirosis, PCR, MAT, Sicilian black swine.

Leptospirosis, caused by the spirochete *Leptospira*, is considered an important reemerging infectious disease worldwide. Leptospirosis is a zoonosis of ubiquitous distribution, caused by infection with pathogenic *Leptospira* species. *Leptospira* have the ability to survive in a wide range of environmental reservoirs, including mammalian hosts.^{1,2} In some regions of the world human leptospirosis outbreaks are an important health problem. In Salvador, Brazil, more than 300 cases of human leptospirosis are identified each year during the rainy season, and 15% of them die.³ In Italy some human leptospirosis cases are reported only in the northern regions mainly connected with contaminated water.⁴ A serological survey by microscopic agglutination test (MAT) on sera samples from northern and central Italy of several animal species showed sero-positive percentages

going from 12.13 % in ovine, 9.46 % in swine to 0.48 % in cattle.⁵ A single limited serological survey had been conducted until now in Sicily on animal leptospirosis (Vesco G et al. Unpublished results). To obtain more data on animal leptospirosis in Sicilian Island we decided to begin a general survey on several animal herds, not only by serological methods (MAT) but also by molecular biology techniques. Conventional identification and diagnosis of *Leptospira* are based on the serologic method of MAT.¹ Numerous PCR-based techniques such as random amplified polymorphic DNA, PCR followed by restriction analysis or hybridization have been developed and evaluated based on typing of leptospiral reference strains.^{1,2,6,7} PCR can be useful also for a rapid diagnosis of leptospirosis particularly in the case of acute human syndromes in which other diagnostic techniques

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can give negative results or are anyway time-consuming.^{1,2} Previously analysis had shown that PCR targeting 16S rRNA gene had a high sensitivity, (M. Vitale et al. unpublished observation). In this paper we focused particularly on the analysis of an autochthon swine population (black swine of Nebrodi) which lives freely in the woods, in conditions very similar to the wild status. The black swine of Nebrodi also called Sicilian is an autochthon Sicilian race of very ancient origin. Today is present in a limited number of animals in Madonie (Palermo) and Nebrodi (Messina) mountains.

METHODS

Isolation has been made from urine samples immediately put on EMJH containing 5-fluoroacil at 100 micrograms/ml; kidney samples were homogenized in the same medium and then incubated as described in OIE manual.⁸

Molecular analysis: DNA was extracted from kidney and liver samples using Fast DNA Kit from Q BIO GENE and Rybolyser Homogenization according to manufacture's instructions.

DNA from urine samples was extracted using 2 aliquots of 200 microliters of urine with Qiagen columns following the manufacture's protocol without any further treatment; at the end we put together the 2 aliquots and perform PCR on 16S rRNA gene. PCR analysis had been performed using 16S rRNA gene as PCR target (primers: LEPTO E1 GGGAAAAATAAGCAGCGATGTG and LEPTO E2-ATTCCACTCCATGTCAAGCC amplicon 571 bp).⁹

The following program in the Applied Biosystem 9 700 thermal cycler was used: 94 °C for 5 minutes 1 cycle followed by 40 cycles at 1 minute at 94 °C, 1 minute at 60 °C, 1 minute at 72 °C. The final extension was at 72 °C for 5 minutes.

DNA from the bacterial strains was extracted by Qiagen column affinity column.

The amplified fragments were visualised on 2 % agarose gel.

RESULTS

PCR method targeting 16S rRNA gene on DNA extracted from tissue and urine samples resulted in a high specific analytic method as shown in figure; pathogen *Leptospira spp.* were detected while several other bacteria species were negative.

We performed analysis on different samples (urine, tissues) from black swine of Nebrodi to look for a prevalence of leptospirosis. In this population of animals we found PCR positive animals in a high percentage up to 40% (Table 1).

The animals were coming from a restricted area in province of Messina in north-east of Sicily in which are located almost 15 herds of these swine with a total population of 900 animals. Black swine are particularly interesting because they live in conditions that are very similar to wild animals and so they can give a better idea of the diffusion of the micro-organism in natural environment.

Survey on tissue and urine samples in bovine and ovine herds coming from the same areas in

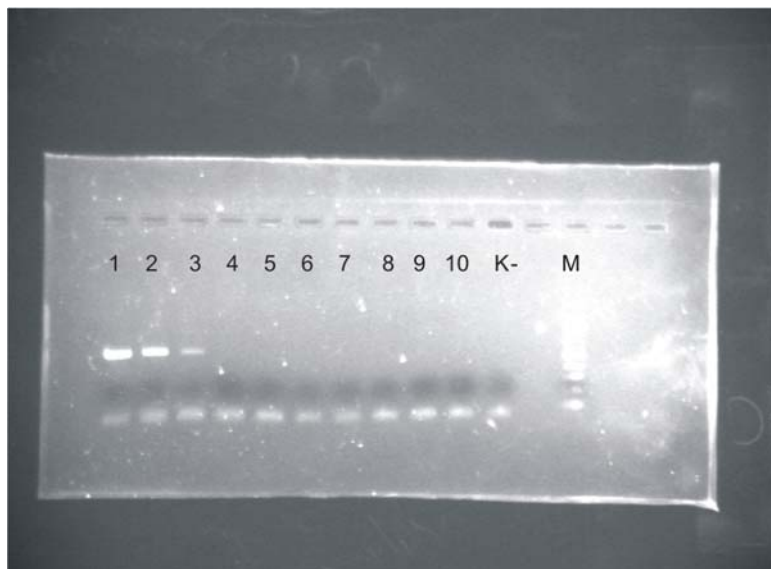


Fig. PCR results on 1. *L.pomoma*; 2. *L.grippothiphosa*; 3. *L.ballum*; 4. *E.coli*; 5. *B. melitensis*; 6. *S. aureus*; 7. *L. patoc*; 8. *S. abortus ovis*; 9. *M. bovis*; 10. *L. infantum*; K-no DNA template; M-100bp ladder.

Sicily showed a lower percentage of PCR and isolation positive animals (Table 2).

TABLE 1. PCR and isolation data on samples from Black swine of Nebrodi

Kidney	PCR +	30/90	33 %
	Isolation +	8/90	8,8 %
Urine	PCR +	28/70	40 %
	Isolation +	15/70	21 %
Liver	PCR +	5/13	38 %
	Isolation +	0/13	

TABLE 2. PCR and isolation data from bovine and ovine samples in Messina province

		Bovine	Ovine
Kidney	PCR +	3/60 (5 %)	3/45 (6,6 %)
	Isolation +	0/60	0/45
Urine	PCR +	5/60 (8,3 %)	3/60 (5 %)
	Isolation +	0/60	0/60

DISCUSSION

The PCR targeting 16S rRNA gene can be a useful tool for the rapid diagnosis of leptospirosis detecting specifically many pathogenic leptospira. We found that the method is quite specific and analysis on DNA extracted by tissue samples and urine gave a higher number of positive animals compared to the isolation procedures. A previous serological screening on 110 bovine herds in south-east Sicily had shown by MAT a prevalence of hardjo serovar up to 29 % on positive farms (Vesco G et al. Unpublished results). Considering that a high percentage of bovine herds (36 %) resulted positive by this method, we decided to plan a new survey to cover the entire island with analysis on different animals. The epidemiological data on animal leptospirosis in Messina province in Sicily showed a high positive percentage by PCR in a population of autochthon swine race (black swine of Nebrodi) while data on other animal herds in the same province showed a lower percentage of positive samples by PCR. The black swine of Nebrodi are freely living in the woods and they are put in restricted area only for a short period right before slaughtering, so it is possible that the higher prevalence is due to their wild living conditions.

ACKNOWLEDGEMENTS

The authors thank to M. Piazza, M. Leonardi and A. Fascetto for their technical assistance. The

work was financed by a grant to M. Vitale "Ricerca corrente 2001" from the Italian Ministry of Health.

Método de reacción en cadena de la polimerasa para la leptospirosis, análisis en muestras de una población autóctona de cerdos en Sicilia, Italia

RESUMEN

Se fijó un método que apuntó al gen del rRNA 16S, que consistió en una sola reacción en cadena de la polimerasa de 40 ciclos que es específica para leptospirosis patógenas. Los resultados negativos de la reacción en cadena de la polimerasa fueron observados en leptospirosis no-patógenas (serovar patoc) y en otras especies de bacterias. Un estudio en una población de manadas de cerdos autóctonos en Sicilia se realizó por este método, particularmente en muestras de riñón de animales sacrificados y en muestras de orina de animales vivos. El análisis demostró una prevalencia de leptospirosis de 40 % en estos animales. En otra manada de bovinos y ovinos, en la misma provincia de Sicilia, se encontró una baja prevalencia.

Palabras clave: Leptospirosis animal, RCP, MAT, cerdos negros de Sicilia.

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Recibido: 27 de diciembre de 2004. Aprobado: 10 de marzo de 2005.

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