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Laboratory investigation of adult small ruminant Leptospirosis, a neglected infection in Greece: problems and recommendations

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Εργαστηριακή διερεύνηση της ελλιπώς μελετημένης στην Ελλάδα λεπτοσπείρωσης των μικρών μηρυκαστικών: προβλήματα και συστάσεις

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ABSTRACT. Leptospirosis is in Greece a neglected infection. Small ruminants and specifically sheep are accidental hosts of *Leptospira spp*, but they could also be disseminators of pathogenic serovars. Thus, the objective was to investigate leptospirosis of adult small ruminants coming from areas in Southern Greece, where accidental evidence had showed that leptospirosis could be an important infection for man and animals. For this purpose, blood and kidney samples were collected at slaughter from adult females. Collected samples were examined with a commercial serological screening kit, the microagglutination test (MAT), histology and PCR. One hundred ten serum and 110 tissue samples were collected. Of the examined serum samples 55 (50%) were suspect for leptospirosis in the screening kit and 28 (25.45%) were MAT positive. Of the tissue samples 38 (34.5%) were PCR positive and 30 (27.2%) showed various degrees of microscopic kidney lesions. The serovars identified by the MAT were Tarassovi (10 animals), Autumnalis (8 animals), Zanon (4 animals), Hebdomadis and Javanica (2 each), Bratislava and Hardjo prajitno (one each). The conclusion is that small ruminants and specifically sheep (98 animals) are disseminators of pathogenic *Leptospira spp.* serovars in areas where they predominate and climatic factors favor the survival of the pathogen.

Keywords: histology, leptospirosis, MAT, PCR, small ruminant

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Περίληψη Η λεπτοσπείρωση στην Ελλάδα είναι μια παραμελημένη μόλυνση. Τα μικρά μηρυκαστικά, ιδιαίτερα τα πρόβατα, αν και θεωρούνται τυχαίοι ξενιστές του γένους *Leptospira*, θα μπορούσαν να γίνουν πηγές διασποράς παθογόνων ορότυπων. Ως εκ τούτου, σκοπός μας ήταν η διερεύνηση της λεπτοσπείρωσης μικρών μηρυκαστικών σε περιοχές της νότιας Ελλάδας, που προηγούμενα ευρήματα έδειχναν ότι η λεπτοσπείρωση αποτελούσε πιθανή σημαντική λοίμωξη των ζώων και του ανθρώπου. Για το σκοπό αυτό οροί αίματος και νεφροί ενήλικων ζώων συλλέχθηκαν κατά τη σφαγή από θηλυκά ζώα. Εκατόν δέκα δείγματα (ορών και ιστών) διερευνήθηκαν με ένα εμπορικό kit ταχείας οροδιερεύνησης, την μέθοδο της μικροσυγκόλλησης (MAT), ιστολογική εξέταση και PCR. Από τα δείγματα ορών 55 (50%) ήταν ύποπτα λεπτοσπείρωσης στο kit and 28 (25.45%) θετικά στη μέθοδο MAT. Από τα δείγματα ιστών 38 (34.5%) ήταν PCR θετικά and 30 (27.2%) είχαν διάφορους βαθμούς μικροσκοπικών αλλοιώσεων. Οι ορότυποι που αναγνωρίστηκαν με τη μέθοδο MAT ήταν Tarassovi (10 ζώα), Autumnalis (8 ζώα), Zanonii (4 ζώα), Hebdomadis and Javanica (2 στον καθένα), Bratislava and Hardjio prajitno (1 στον καθένα). Συμπεραίνεται ότι τα μικρά μηρυκαστικά και συγκεκριμένα τα πρόβατα (98 από τα 110 ζώα) είναι πηγές διασποράς παθογόνων ορότυπων *Leptospira spp.* Σε περιοχές που οι κλιματολογικές συνθήκες ευνοούν την επιβίωση του παθογόνου.

Λέξεις κλειδιά: ιστολογία, λεπτοσπείρωση, MAT, μικρά μηρυκαστικά, PCR

INTRODUCTION

The systematic investigation of animal leptospirosis across the world depends on each government's ability to finance national disease surveillance. Some nations include leptospirosis in the list of diseases with significance for public health (Biosecurity Australia, 2001; Sambasiva et al., 2003; Jansen et al., 2005; Zhang et al., 2012). Others have yet to recognize its importance and systematically investigate the infection (Hartskeerl et al., 2011). Thus, international knowledge on the spread of leptospirosis and the serovars involved in animal and human infections is contributed by those systematically investigating leptospirosis. They report prevalences from man and animals reaching 90% in tropical regions (Kawaguchi et al., 2008; Zhang et al., 2012).

Greece is not among the states systematically investigating leptospirosis. Thus, there are few published contributions. They are reporting prevalence rates from 5.7 to 24.9%, depending on the clinical history of the examined animals and geographic area of their origin (Burriel et al., 2002; Burriel et al., 2003; Bisias et al., 2010).

The reported prevalence values across the world and the predominant serovars are deriving from the use of the microagglutination test (MAT), an internationally recognized serologic method (the gold

standard) for investigating animal infections (ILS - WHO, 2003; Levett, 2004; OIE, 2008; Hartskeerl et al., 2011). The method uses live *Leptospira spp* serovars, thus it requires the maintenance of a large set of serovars (over 20) needing weekly subculturing by knowledgeable and dedicated scientists. On addition, a positive result in the MAT does not always associate to active infection, thus examination of paired serum samples is necessary (OIE, 2008). However, the MAT is the best available serologic method for serovar specific information, hence helping to accurately record the predominant serovars in an area or country. This is the reason the method is to this day recognized as the best official method for testing serum from animals and man regardless of stage of infection. What should be noticed, however, is that if a serovar is not included in the set of serovars for testing against it, it will not be recorded as present in an area (Levett, 2004; OIE, 2008; Cerqueria and Picardeau, 2009; Hartskeerl et al., 2011).

Other serologic methods used for investigating leptospirosis lack specificity and sensitivity as to involved serovars (Levett, 2004; OIE, 2008; Cerqueria and Picardeau, 2009) or need animal and serovar specific reagents, which are not commercially available or cannot be easily produced (Croda et al., 2007; Dounngchauwee et al., 2008; Saglam

et al., 2008). For the majority of important bacterial infections, isolation and identification of the causative agent is the confirmation and, in most cases, it is quicker than paired serum samples. Unfortunately, this is not the case with *Leptospira spp.* (Levett, 2004; OIE, 2008; Hartskeerl et al., 2011).

Isolation for confirmation of leptospirosis is difficult, time consuming, expensive and requires a well organized reference laboratory (Levett, 2004; OIE, 2008; Hartskeerl et al., 2011). Hence, nations considering the pathogen of secondary public health importance do not finance its systematic study due to costs. Similar difficulties are faced when using methods for indirect recognition of the microorganism's presence in tissue or methods molecularly identifying it in body fluids and tissue samples. Problems result from either lack of commercially available reagents or lack of costly technology (Dounngchauwee et al., 2008; Lilenbaum et al., 2008; Saglam et al., 2008; Lilenbaum et al., 2009).

With these difficulties in mind and lack of state support, the ambitious objective of the present work was to evaluate small ruminant leptospirosis in association to serologic identification of positive animals, kidney lesions and the confirmation of the pathogen's presence in tissue using PCR, staining and isolation.

MATERIAL AND METHODS

Collection of serum and tissue samples

After a preliminary serological investigation of infectious abortion causes in small ruminants (Bisias et al., 2010) in the province of Peloponnesus Southern Greece, two areas were identified as having the highest probability of isolating *Leptospira spp* from small ruminants. Female adults arriving to two slaughter houses of the two selected areas were bled before slaughtering and their kidneys were removed from the carcass by the meat inspectors. Kidneys immediately packed in ice and serum samples were sent in Athens by public transport. As soon samples were received, kidney surface was sterilized by dipping in clinical alcohol, flamed and aseptically dissected. Tissue sections were selected from areas with

macroscopic or suspect for microscopic lesions associated to the presence of leptospira microorganisms. Selected tissue sections collected from both kidneys of each animal were divided in three parts. One was immediately prepared for culturing, one was placed in a sterile plastic universal and frozen in -80°C and the third was put in 10% formalin solution. All serum samples collected were kept in -80°C for later use.

Isolation of *Leptospira spp*

Isolation was attempted and financially supported by the Public Health Veterinary Laboratory of the Athens School of Hygiene. The attempted isolation of *Leptospira spp* followed the guide lines of the OIE Terrestrial Manual (2008) using the commercially available culture media Ellinghausen–McCullough–Johnson–Harris (EMJH) (Becton Dickinson Hellas). Selected tissue sections from each animal were aseptically homogenized by stomacher (easymix, AES Laboratories, France). Dilutions up to 1/1000 were prepared and filtered with 0.45 µm filters (Merck, Germany). Two to three drops from each filtered dilution were inoculated into EMJH medium with or without 5-Fluorouracil and incubated at 29±1°C for up to four months.

Serologic Investigation

One hundred ten serum samples kept in -80°C were split into two aliquots. Two serologic methods were used. Thus, one aliquot was sent for testing by the Institute of Infectious and Parasitic Diseases, Centre of Athens Veterinary Institutions, Greece. They were tested with a rapid agglutination screening kit (*Leptospira* Serology, BIO-RAD, France) used for screening human sera. Any evidence of agglutination was recorded regardless of the kit's instructions of what is a positive sample. The other aliquot was sent by courier to the National Veterinary Laboratories Agency (NVAL) of the UK. Here the Standard Operating Procedures of the MAT using 19 live serovars belonging to six serogroups was used for testing the mailed 110 serum samples. A positive serum sample agglutinated 50% of the chosen live serovars at a dilution of 1/100.

PCR detection of *Leptospira* spp in tissue

PCR was performed by the Laboratory of Microbiology and Parasitology, Faculty of Veterinary Medicine, University of Thessaly, according to procedures published by Kwok and Higuchi (1989) and Gravenkamp et al. (1993), with small modifications. Frozen sections were defrosted and small tissue sections (2-3) were removed and prepared for PCR following the protocol for DNA purification from tissue, published by Puregene (Gentra Systems, USA). For the multiplex PCR the commercial kits Puregene (Gentra Systems, Minneapolis Minnesota, USA) and Invitrogen (Invitrogen, Carlsbad, CA, USA) were used with reagents supplied from the same suppliers. The two pairs of primers used were, pairs G1 5'-ctgaatcgctgtataaaagt-3' / G2 5'-ggaaaacaaatggctcggaag-3' and pairs B64-I 5'-ctgaattcatctcaactc-3' / B64-II 5'-gva-gaaatvagatggacgat-3'.

They are identifying pathogenic species of *Leptospira*. One hundred ten tissue samples were examined. The positive control was *L. interrogans*, seovar Icterohaemorrhagiae and the negative water with DEPC.

Histological evaluation of Kidney tissue

Two to three tissue blocks from each animal were sectioned for histological examination. A total of 263 blocks were sectioned and stained with hematoxylin – eosin stains (IVD, Merck, Greece). The staining method was according to the working protocol of Fischer et al., (2008). Kidney sections microscopically having evidence of lesions possibly associated to leptospira microorganisms were prepared for staining by the Steiner Modified Silver Stain Kit (Sigma-Aldrich, Greece) following the recommended protocol of Newcomer Supply Laboratory, USA.

RESULTS

Isolation

Twenty four kidney tissue samples from 24 animals were cultured. The attempted isolation did not yield any positive results during a four month trial

period. Thus, the hosting laboratory withdrew its financial support before the completion of the project.

Serologic Investigation

Fifty five (50%) serum samples showed evidence of agglutination (light diffuse or peripheral partial agglutination) by the rapid screening kit. These samples were characterized only as suspect due to lack of agglutination in the degree suggested by the working protocol of the kit.

Twenty eight (25.45%) serum samples were positive to the MAT (NVLA, UK) at titers of 1/100 to 1/800. Five of them had positive titers to two serovars. Fifteen (53.5%) were also suspect with the commercial rapid kit. The serovars identified by the MAT were Tarassovi (10 animals), Autumnalis (10 animals), Zanon (6 animals), Hebdomadis and Javanica (2 each), Bratislava and Hardjio prajitno (one each).

PCR detection of *Leptospira* spp in tissue

Thirty eight (34.5%) tissue samples were positive with the multiplex PCR. Of them 23 (60.5%) were also positive with the MAT and 17 (56.6%) were suspect with the rapid screening kit, but only 8 (21%) were positive with all three methods.

Histological evaluation of Kidney tissue

Thirty (27.2%) animals showed mixed microscopic evidence of kidney damage ranging from mononuclear interstitial infiltrations (18 animals), interstitial fibrosis (9 animals), mild glomerulonephritis (17 animals) and mild tubular atrophy (16 animals). Of the 30 animals, 20 (66.6%) had a positive PCR, 18 (60%) were from MAT positive animals and 10 (33.3%) had a suspect rapid screening kit. Furthermore, 16 (53.3%) were positive in both the MAT and PCR, but only 5 (16.6%) were positive in all four methods. Six (20%) tissue samples of those having histological lesions showed evidence of microorganisms present in tissue sections. Four of them had MAT titers between 1/200 and 1/400 and they were also positive to PCR.

DISCUSSION

Various problems developed during the present investigation due to lack of state supported laboratory facilities to successfully investigate the pathogen. The most important problem faced was the decision of the Public Health Veterinary Laboratory of the Athens School of Hygiene to withdraw its support for isolating the pathogen, due to costs. This decision is evidence of the low National priorities on the zoonotic agent *Leptospira* spp in Greece. Other problems were the complete lack of state support of this research making sampling and sample delivery extremely difficult and time consuming. Thus, the failing of isolating the pathogen could be caused by the time elapsed between tissue collection and attempted isolation, although seven of the 24 animals examined by isolation were PCR positive and four of them were also MAT positive at titers 1/100 and 1/200.

The MAT results showed that leptospirosis was subclinically present, but due to the very small number of goat samples, a comparison between the two species was impossible. Previous reported serologic investigations in Greece showed that the prevalence between sheep and goats not having evidence of clinical disease significantly differs (5.7 Vs 16.2 respectively) (Burriel et al., 2003). However, when serum samples are examined from sheep flocks and goat herds with a history of abortion the reported prevalence is found similar (13.6 vs 12.4 %) (Burriel et al., 2002). In the work preceding the present investigation and examining serum samples from confirmed abortion cases from high risk areas, goats appeared more resistant to infection (18.4%) compared to sheep (24.9%) (Bisias et al., 2010) and this is in agreement with the findings of others (Lilenbaum et al., 2010). Unfortunately, in the current investigation the number of goats was very small for their comparison with sheep.

Significant differences were also observed on the reported serovars between previous investigations in Greece and the present. Previously reported serovars predominant in sheep were Bratislava with Australis second and in goats of equal importance Bratislava and Copenhageni (Burriel et al., 2003). In the report

preceding the present and concerning abortion cases (Bisias et al., 2010) significant serovars for sheep were Tarrasovi, Australis and Bratislava and for goats Australis, Tarassovi and Copenhageni. In the present report, the common characteristic of all samples was their origin from high risk areas, thus explaining the observed high proportion (25.4%) of MAT positive animals without any clinical evidence of infection. In addition, the predominant serovars differed from previous investigations (Burriel et al., 2002; Burriel et al., 2003), but they were closer to those from the preliminary investigation (Bisias et al., 2010) between abortion cases. They were serovar Tarassovi of the species *L. borgpetersenii* and Autumnalis and Zanoni of the species *L. interrogans* (Sakolvaree et al., 2007; Cerqueria et al., 2010). All three considered pathogenic for man (Biosecurity Australia, 2001).

If past and present results from Greece are compared, when defining the prevalent serovars, it becomes evident, that there is a need for systematically investigating the infection using the MAT. Such knowledge is required for evaluating the need of a vaccination program for small ruminants in high risk areas. Because vaccines confer best protection only to homologous serovars (ILS-WHO, 2003; Wang et al., 2007; Cerqueria and Picardeau, 2009; Hartskeerl et al., 2011), knowledge of the predominant serovars will determine the success of commercially available vaccines. Nevertheless, a positive MAT does not indicate active infection, thus it requires confirmation with other available methods or means for establishing active infection. One such method is isolation, but due to its time limitations and the time required for examining a second serum sample, various PCR versions have been established for quickly confirming clinical leptospirosis (Gravenkamp et al., 1993; Bomfim and Koury, 2006; Lilenbaum et al., 2008; Lilenbaum et al., 2009). However, comparisons between PCR, culture and serologic results are not always satisfactory (Faber et al., 2000; Soto et al., 2006; Barbante et al., 2014), if infecting serovars are not included in the MAT testing. In such cases, PCR could be positive, but the MAT negative. Thus, the largest the number of serovars included in the

MAT the greater should be its sensitivity and agreement with the results of a PCR.

In the present investigation, from the 38 tissue samples positive with the PCR only 23 were also positive with the MAT. PCR appeared as more sensitive compared to the MAT, as others report (Bomfim and Koury, 2006; Lilenbaum et al., 2008; Barbante et al., 2014), but there is also a possibility that the group of serovars used in the MAT for testing in the UK is not suitable for Greece. Therefore, some MAT negative animals could be positive in serovars not included in the testing panel. Perhaps, a different set of serovars could have shown a better agreement between the two methods and could also have changed the predominant serovars, thus decisions on a vaccine. Another possibility is that PCR false positives were contributing to the observed differences and this could have been clarified with successful isolation, histochemical staining or the visualization of the pathogen in tissue using transmission electron microscopy (Hamir et al., 2001; Szeredi and Haake, 2006; d'Andon et al., 2014). One PCR problem, which is also a problem for other methods used for screening animals or confirming human cases is lack of serovar recognition, which is the major advantage of the MAT method (Levett, 2004; Dounghawee et al., 2008; Saglam et al., 2008; Lilenbaum et al., 2009; Hartskeerl et al., 2011). Until serovar recognition by molecular methods becomes possible, the MAT using a large number of serovars will remain the preferred method for epidemiologically investigating animal leptospirosis.

Of the 30 animals having microscopic evidence of kidney lesions, 20 (66.6%) were also positive in the PCR, an agreement similar to that of the MAT and PCR (60.5%). If the MAT and PCR findings

are considered evidence of *Leptospira spp* presence in kidney tissue during the life of the animal, then increased is the possibility that the observed microscopic lesions were caused by the presence of the pathogen. The last is strongly supported by the observations that 16 (53.3%) of those having histological lesions were positive in the MAT and PCR. Experimentally, the same lesions are observed in chronic infection (d'Andon et al., 2014) supporting our present hypothesis, that observed lesions are resulting from the chronic colonization of kidney tissue by leptospira, although it was not confirmed here by isolation or visualization of the pathogen in all the samples. The modified silver staining method was developed many decades ago (Blenden and Goldberg, 1965). Today, it uses commercially stabilized reagents, but its use in the present work did not show evidence of the pathogen for the majority of the tissue with lesions. Perhaps, the reason of failing to visualize the pathogen was the low numbers of leptospiral cells in the examined tissue or their absence at the time of staining. Accidental hosts of *Leptospira spp.*, like sheep, become chronically infected, but the pathogen is intermittently reaching high numbers in urine, thus tissue (Monahan et al., 2009). In some other cases, the infecting serovar may persist for longer, if it has adapted to its host (Ahmed et al., 2012), thus be visualized by staining. Sheep, considered an accidental host, is, perhaps, eliminating quicker some of the pathogen's serovars.

Nevertheless, the results obtained by this investigation are evidence that sheep (the majority of the sampled animals) are not only accidental hosts of the pathogen. They maintain, in high risk areas, serovars of the pathogen, thus, becoming an important reservoir of serovars potentially pathogenic to man and animals. ■

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