Detection of contagious caprine pleuropneumonia in East Turkey

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Summary
A study was implemented to investigate the presence of contagious caprine pleuropneumonia (CCPP) in East Turkey. This study was based on clinical surveillance in the field, surveillance at regional slaughterhouses and regular submission of suspected lesions to regional laboratories. The results showed that the agent of CCPP, *Mycoplasma capricolum* subspecies *capripneumoniae* (*Mccp*), could be detected by culture and specific polymerase chain reaction from 37.5% (12/32) of lung samples taken from goats of ten different herds. This agent was also isolated from two of 13 sheep samples (one from the lung and the other from a nasal swab). *Mycoplasma capricolum* subsp. *capripneumoniae* was isolated in pure culture and characterised at a finer molecular level. The East Turkish isolate was found to be closely related to another strain of Turkish origin, as well as to *Mccp* strains isolated in Tunisia. The isolation of *Mccp* from sheep lung lesions brings the strict host-specificity of this pathogen into question. It may also indicate that *Mccp* presents a risk for wildlife in the region. Such results, the authors believe, demonstrate that adequate risk assessments should be undertaken in Turkey and neighbouring countries.

Keywords

Introduction

Contagious caprine pleuropneumonia (CCPP) is a devastating disease of goats, associated with infection by *Mycoplasma capricolum* subspecies *capripneumoniae* (*Mccp*). The disease is clinically characterised by fever, coughing and respiratory distress, with associated lesions, such as fibrinous pleuropneumonia, unilateral hepatisation of one lung and accumulation of pleural fluid in the thoracic cavity (15). The disease is included in the list of notifiable diseases of the World Organisation for Animal Health (OIE) because of its high morbidity and mortality, significant economic impact on livestock and the restrictions on trade caused by CCPP once it has been declared present in a country. The disease threatens a significant number of goat populations throughout the world, with a high impact in Africa and Asia, including Turkey. *Mycoplasma capricolum* subsp. *capripneumoniae*, formerly known as *Myoplasma* sp. type F38, was isolated for the first time and characterised only 30 years ago (6).

Of the *Mycoplasma mycoides* cluster, *Mccp* is the most difficult to grow in vitro, which partially explains its lack of widespread distribution. The recent development of a specific polymerase chain reaction (PCR) has made the detection of *Mccp* much simpler (19). Furthermore, PCR products can be sequenced, offering the possibility of
typing the strains at a finer level and allowing molecular epidemiological studies (8).

The occurrence of CCPP in Turkey has been suspected for a long time, but all previous trials to isolate the CCPP agent were unsuccessful (5), and the mycoplasmas that were isolated (4) belonged to other species of the *Mycoplasma mycoides* cluster. The first isolation of *Mcpp* in Turkey was reported in 1990 (17). In addition, CCPP was clearly demonstrated in 2005 when the disease occurred in the Thrace region of Turkey (14), threatening neighbouring countries in the European Union. At that time, control measures consisted mainly of antibiotic treatments (13).

According to data obtained from the Turkish Statistical Institute, in the year 2004, the goat population of Turkey was approximately seven million, 12% of which were found in East Turkey (18). In recent studies conducted in Thrace, *Mcpp* has been isolated from goat lung and pleural fluid samples (12). In the same region, serological testing of blood samples from animals that were suspected of being infected revealed that approximately 50% tested positive for the presence of antibodies against CCPP (12). Although the presence of the disease has been confirmed in Turkey by isolation of the causative agent, information on the prevalence and exact distribution of CCPP is lacking.

This study was conducted to confirm unequivocally the presence of CCPP in East Turkey, by isolating the causative agent and characterising it through molecular tools.

### Materials and methods

#### Field survey

A field survey was conducted in the main towns around Elazig, which is located in eastern Turkey, for more than a year (from February 2006 to May 2007). Samples were taken from 24 herds of goats and 13 flocks of sheep in six towns:

- Malatya
- Elazig
- Bingol
- Bitlis
- Mus
- Siirt.

All these towns are well distributed throughout the east of the country (Fig. 1).

The area from which the samples originated encompasses more than 50,000 km². Veterinarians from the Turkish Ministry of Agriculture were asked to report any suspicion of CCPP that they might encounter during their field trips. Local abattoirs were also contacted and asked to report and take samples from pneumonic lesions observed in sheep and goats. In addition, staff from Fırat University and the Veterinary Control and Research Institute in Elazig visited these abattoirs once a week. Most samples were collected in winter, between mid-November and the end of March.

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**Fig. 1**

*Map of East Turkey showing locations of contagious caprine pleuropneumonia outbreaks*

The various locations where outbreaks of contagious caprine pleuropneumonia were confirmed have been indicated with red dots. This map also shows the neighbouring countries which were at risk.
Samples collected in the field were then submitted to the local Veterinary Control and Research Institute, which is responsible for monitoring disease in the 13 provinces of the region. Lung samples, collected either from the abattoirs or from animals autopsied at the University, were submitted to the Department of Microbiology, Firat University. Pleural fluid samples were also collected from four goats autopsied in the Pathology Department. In addition, two nasal cavity swabs (one from a live goat and one from a live sheep) were collected from CCPP-suspected animals in the field. All samples were immediately transported to the laboratory and subjected to culture or kept at –20°C, until required.

Isolation of Mycoplasma

Lung, pleural fluid and nasal swab samples were processed according to the method described by Thiaucourt et al. (16). In summary, Mccp was isolated by both serial dilution in modified Hayflick medium (pleuropneumonia-like organisms [PPLO] broth without crystal violet [21 g/l], 20% de-complemented horse serum, 10% fresh yeast extract, 0.2% glucose, 0.4% sodium pyruvate, 0.04% ampicillin) and by streaking onto solid PPLO agar of the same medium, simultaneously. The broth medium was solidified by adding 1% agar noble to the medium. Samples were diluted by five subsequent 10-fold dilutions. The lung samples (1 cm³) were added to the first broth medium tube (5 ml) and mixed properly. Next, 300 µl broth was taken from each tube and mixed. The samples were incubated at 37°C in 5% CO₂ for 7 to 10 days. The broths were checked daily and the samples with growth (indicated by turbidity in the broth cultures) were subcultured onto agar plates from the last turbid tube. The plates were checked daily for the appearance of colonies. The isolated colonies were inoculated into the stock suspension (50% broth/50% horse serum) and kept at –20°C. Incubations from a loopful of pleural fluid and nasal swab samples were also carried out following this procedure.

Detection and identification of Mycoplasma capricolum subspecies capripneumoniae by polymerase chain reaction

Positive cultures in liquid broth were centrifuged at 12,000 g for 20 min and the pellet was re-suspended in 300 µl of water. The bacterial suspension was then treated with 300 µl TNES buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 10 mM tris-ethylenediamine tetra-acetic acid, 0.2% sodium dodecyl sulphate) and proteinase K (200 µg/ml), and kept at 56°C for 1 h. The suspension was heated at 95°C for 10 min to inactivate proteinase K. Two different PCR procedures were applied. In the first, deoxyribonucleic acid (DNA) samples were tested with group-specific PCR for Mycoplasma spp. (3), and, in the second, they were tested with subspecies-specific PCR for Mccp (19).

The PCRs were performed in a TC 512 Temperature Cycling System in a reaction volume of 50 µl, containing 5 µl 10 × PCR buffer (750 mM Tris HCl, pH 8.8, 200 mM (NH₄)₂SO₄, 0.1% Tween 20), 5 µl of 25 mM MgCl₂, 250 µM of each deoxynucleotide triphosphate, 1.25 U Taq DNA Polymerase, 20 pmol of each primer and 25 ng of template DNA. Primer pairs specific to the Mycoplasma genus (GPO3F 5’-TGGGGACCAAAAGGATTAGATTACCC-3’ and MGO 5’-TGCACCATTGTCACTCTGTTAAC CTC-3’) and specific to Mccp (Mccp-spe-F 5’-ATCAT TTATAATCCCTTCAAG-3’ and Mccp-spe-R 5’-TACT ATGAGTAAATTATATATGCAA-3’) were employed.

The reaction conditions for the group-specific PCR were as follows:
- one cycle of the denaturation step at 94°C for 2 min
- 35 cycles of denaturation at 94°C for 15 s
- annealing at 53°C for 15 s and extension at 72°C for 15 s
- one cycle of the extension step at 72°C for 5 min.

In the Mccp-specific PCR, following an initial denaturation step at 94°C for 2 min, amplification was obtained with:
- 35 cycles of denaturation at 94°C for 30 s
- annealing at 47°C for 15 s and extension at 72°C for 15 s
- a final extension step at 72°C for 5 min.

The amplified products were detected by staining with ethidium bromide (0.5 µg/ml) after electrophoresis at 80 volts for 2 h in 1.5% agarose gels. Polymerase chain reaction products with a molecular size of 280 base pairs (bp) and 316 bp were considered indicative for Mycoplasma spp. and Mccp, respectively.

A vaccine strain of Mccp (GL 102) was included as a positive control and distilled water was used as a negative control in all assays.

Molecular typing of the Mycoplasma capricolum subspecies capripneumoniae strain

One Mccp strain sent to the French Agricultural Research Centre for International Development (CIRAD) laboratory, the Reference Laboratory of the OIE and Food and Agriculture Organization of the United Nations (FAO), was further characterised. First, its identity was confirmed by partially sequencing five housekeeping genes: fusA, lepA,
gyrB, rpoB and glpQ. These sequences were then compared to others to establish a more precise phylogenetic analysis of mycoplasmata belonging to or related to the *Mycoplasma mycoides* cluster (9).

The same strain was used as a template for the amplification of a 2,400 bp-long fragment, used to perform molecular epidemiology studies of CCPP (8).

**Results**

**Field survey**

Contagious caprine pleuropneumonia was suspected in 24 herds, which comprised 1,122 animals. Within these herds, 459 animals (41%) showed respiratory distress. Of these, 89 died (a 19% fatality rate), in spite of the usual antibiotic treatments (tylosin and fluoroquinolones), given in cases of clinically evident respiratory disease.

**Bacteriological findings**

Bacterial growth, as indicated by turbidity in the broths and ‘fried egg’ colonies in the agar plates, was observed in 25 (22 from goats and 3 from sheep) out of 45 samples (55.6%), submitted for bacterial investigation. In addition, colonies typical of *Mycoplasma* were obtained from these samples when the broths were inoculated onto solid media. In the bacteriological examination of the lung lesions, growth was seen in 21 (67.7%) of 31 goat samples and two (16.7%) of 12 sheep samples. While 15 of the goat lung samples with growth were provided by the local institute, three came from the Pathology Department and three were from local abattoirs. Of the two positive sheep lung samples, one was obtained from the local institute and the other from the abattoirs. Moreover, two nasal swab samples (one from a goat and one from a sheep) were determined to be positive for culture (Table I).

Growth was seen in both the lung and pleural fluid samples of the three goats from the Pathology Department. These animals showed marked pleuritis and pleural effusion at necropsy. The pleural fluid was straw-coloured and not viscous. A fine granular texture with hepatisation was also observed in the cut surface of the lungs.

The isolates were identified as *Mycoplasma* spp. when the plates were examined under a stereoscopic microscope for ‘fried egg’ colonies.

**Polymerase chain reaction findings**

The total of 25 culture-positive isolates, including two nasal cavity samples, were confirmed as belonging to the *Mycoplasma* genus by the group-specific PCR, which produced specific bands with the molecular size of 280 bp. In the *Mccp*-specific PCR amplification of the samples, 12 of the goat isolates (all lung samples) and two of the sheep isolates (one nasal cavity sample) produced positive products with the approximate molecular size of 316 bp (Fig. 2). The isolation percentages of *Mccp* were therefore calculated as 37.5% (12/32) in goats and 15.4% (2/13) in sheep (Table I). When the results were considered at the herd/flock level, it was found that *Mccp* was isolated from 10 different goat herds and two sheep flocks.

**Molecular typing of the Mycoplasma capricolum subspecies capripneumoniae strain**

Concatenated sequences of the five housekeeping genes for the East Turkish *Mccp* isolate were identical to sequences obtained from other *Mccp* strains, isolated in Ethiopia.
Kenya, Tunisia and Oman. The references of the corresponding sequences and accession numbers are as follows:
– fusA, EF071739
– glpQ, EF071765
– gyrB, EF071791
– lepA, EF071817
– rpoB, EF071843.

This confirms, once again, the identity of the Turkish Mccp strain.

The sequence of the H2 locus for the East Turkish Mccp isolate was found to be strictly identical with that found in sequences from strains isolated in Tunisia, as well as from the other Turkish strain isolated in Oman. The corresponding sequence accession number is AF378157. This result confirms the close relationship between Turkish Mccp isolates and those from North Africa.

Discussion and conclusion

Although the presence of CCPP has previously been acknowledged in Turkey (17), the disease has not been paid enough attention in this country. The main reason for this is the inadequacy of laboratory facilities and lack of experienced personnel, since the causative agent is rather difficult to cultivate in vitro. So far, only a few studies have been conducted by the scientists working at the Pendik Veterinary Control and Research Institute in Istanbul. They reported that CCPP was observed in the goat population of western Turkey (14). However, no quantitative data are available for the potential presence of CCPP at the national level in Turkey, and it is therefore difficult to estimate the degree of threat that it poses to the national goat population. On the other hand, it is well known, from a significant number of studies of pneumonia and mastitis in small ruminants, that other Mycoplasma spp., such as M. mycoides subsp. capri, M. agalactiae and M. arginini, have been reported as widely present all over the country (10, 11, 20). This raises the possibility that outbreaks due to Mccp might well have gone undetected.

The present study was performed to provide information on whether CCPP occurs in small ruminants in eastern Turkey.

The type of surveillance that was put in place for this purpose proved very efficient, namely, a strategy including:
– field surveillance with veterinarians and technicians
– regular abattoir surveillance
– rapid examination of suspected samples in a laboratory capable not only of isolation but, more importantly, direct detection by PCR.

The CIRAD laboratory then rapidly confirmed the identity of the isolated Mycoplasma strains. This experience shows that the distribution of CCPP could be better and more
rapidly assessed if such a strategy were always implemented where CCPP was suspected. Conventional bacteriological tests were not employed in this study, due to some well-known drawbacks, such as time consumption, difficulty in interpretation and erroneous results due to bacterial contamination.

Although goats were the most affected by CCPP, *Mccp* has also been isolated from a significant number of sheep with pneumonic lesions. This is not the first time that this has been reported (7), which raises questions over the host specificity of *Mccp*. In fact, CCPP has already been characterised in species other than domestic goats and sheep. It has also been found in such wildlife species as the wild goat (*Capra aegagrus*), generuk (*Litocranius walleri*) and Laristan mouflon (*Ovis orientalis laristanica*) (1, 2). The latter species is closely related to sheep, being of the same genus. Furthermore, Laristan mouflons are found in the north of Iran, not very far from East Turkey, where the two countries share a 560 km border. These findings indicate the need for more detailed epidemiological studies of CCPP, the role that sheep can play in its transmission or persistence, and the threat it may pose to endangered wildlife species.

In many cases, the isolation of *Mccp* in pure culture has been hindered by the presence of *M. ovipneumoniae*, which grows at a faster rate than *Mccp*. This did not prevent the detection of *Mccp* in the primary cultures by PCR, and the confirmation of CCPP. However, the regular isolation of *M. ovipneumoniae* may call for renewed research on the possible pathogenicity of this species, which is usually considered as saprophytic.

Although the number of samples examined in this study was small, the isolation of *Mccp* in 37.5% of goats in ten different herds located in geographically distinct areas of eastern Turkey calls for attention. When the results of the present study are considered together with those conducted quite recently in the west of the country, and those from the literature, it seems plausible to suggest that CCPP is a national, or even regional, rather than localised, problem in East Turkey. Owing to the uncontrolled nature of animal movements in this region, the presence of CCPP in neighbouring countries (Georgia, Armenia, Azerbaijan, Iran, Iraq and Syria) should also be suspected. This points to the need for additional enquiries to establish the nature of the threat posed by CCPP to domestic and wild ruminants in the whole of Turkey, as well as in the Thrace region. Without such studies, it will not be possible to adopt adequate and much-needed control measures.

The current vaccines produced in Turkey do not contain an *Mccp* strain. However, they do contain a strain (BQT) which belongs to the *Mycoplasma mycoides* cluster. It is therefore questionable if the vaccine actually protects against CCPP and comparative efficacy trials are urgently needed to address this issue. Unfortunately, CCPP vaccines are inactivated and adjuvanted, which makes them much more expensive than attenuated vaccines, such as those used for controlling contagious bovine pleuropneumonia. At present, only a few vaccine plants are producing such vaccines and producers face difficulties in meeting even the local needs in Kenya and Ethiopia. It is therefore unlikely that these vaccines will be widely used in the field and it is also unlikely that CCPP will be controlled, without strong action by the State and Veterinary Services.

One possible way to lessen the costs of CCPP vaccination could be to design combined vaccines that include other components, such as *Brucella melitensis* antigen, or *Bacillus anthracis* vaccine spores, to combat these important Mediterranean zoonoses at the same time. Such vaccines could have a regional impact, as CCPP and brucellosis have also been reported in North Africa. Furthermore, the molecular characterisation of Turkish *Mccp* strains shows that they are closely related to *Mccp* strains isolated in Tunisia 25 years ago. This should ensure that such vaccines would also be effective in North Africa.

As a whole, the findings of the authors confirm that CCPP is widespread in Turkey. Further epidemiological enquiries are needed to establish the nature of the threat posed by CCPP to domestic and wild ruminants in the whole of Turkey, as well as in the Thrace region. Without such studies, it will not be possible to adopt adequate and much-needed control measures.

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Détection de la pleuropneumonie contagieuse caprine en Turquie orientale

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Résumé
Les auteurs présentent les résultats d’une étude sur la présence de la pleuropneumonie contagieuse caprine (PPCC) en Turquie orientale. À cette fin, une surveillance clinique a été exercée sur le terrain, ainsi qu’une surveillance des abattoirs régionaux ; les lésions suspectes ont été systématiquement envoyées aux laboratoires régionaux pour analyse. Lors de cette étude, l’agent causal de la PPCC, *Mycoplasma capricolum* sous-espèce *capripneumoniae* (*Mccp*) a été détecté par culture et amplification en chaîne par polymérase spécifique dans 37,5 % (12/32) des échantillons de poumon prélevés sur des chèvres provenant de dix cheptels différents. L’agent a également été isolé dans 2 des 13 échantillons ovins analysés (un échantillon de poumon et un écouvillon nasal). *Mycoplasma capricolum* ssp. *capripneumoniae* a été isolé en culture pure ; une caractérisation moléculaire plus précise a ensuite été réalisée. Cet isolat de Turquie orientale est très proche d’une autre souche d’origine turque, ainsi que des souches de *Mccp* isolées en Tunisie. L’isolement de *Mccp* dans des lésions pulmonaires de mouton remet en cause la spécificité d’hôte de cet agent pathogène. Il alerte également sur le risque que *Mccp* peut représenter pour la faune sauvage de la région. Les auteurs considèrent que ces résultats devraient inciter à conduire des analyses du risque appropriées en Turquie et dans les pays voisins.

Mots-clés

Detección de la pleuroneumonía contagiosa caprina en Turquía oriental

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Resumen
Este estudio, dirigido a investigar la presencia de la pleuroneumonía contagiosa caprina en Turquía oriental, se basó en la vigilancia clínica en el terreno, la vigilancia en los mataderos regionales y el envío sistemático de las lesiones sospechosas a laboratorios regionales. Los resultados mostraron que *Mycoplasma capricolum* subespecie *capripneumoniae* (*Mccp*), el agente de la pleuroneumonía contagiosa caprina, podía detectarse por cultivo y mediante la técnica de la reacción en cadena de la polimerasa específica en el 37,5% (12/32) de las muestras pulmonares de cabras provenientes de 10 rebaños diferentes. También se lo aisló en una muestra pulmonar y un hisopo nasal de un grupo de 13 muestras de ovinos. *Mccp* se detectó por cultivo puro y se procedió a su caracterización molecular detallada. El aislado de Turquía oriental presentaba una estrecha afinidad con otra cepa turca, así como con cepas de *Mccp* detectadas en Túnez. El aislamiento de *Mccp* en lesiones pulmonares ovinas
pone en entredicho la estricta especificidad del hospedador de este patógeno. También podría indicar que constituye un riesgo para los animales salvajes de la región. En opinión de los autores, los resultados demuestran la necesidad de efectuar evaluaciones de riesgos apropiadas en Turquía y los países vecinos.

**Palabras clave**

**References**


