Chlamydophila psittaci and Chlamydophila pecorum infections in goats and sheep in Egypt

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Summary
The aim of this study was to investigate the epidemiology of chlamydiosis in free-ranging asymptomatic and diarrhoeic sheep and goats in Egypt. Faecal swabs were examined for the presence of Chlamydiae by culture in Vero cells and chick embryos, and staining with Giménez, direct fluorescein-conjugated monoclonal antibodies, and immunoperoxidase. Specific chlamydial DNA was identified by amplification of the omp2 gene. The asymptomatic goats were 50% positive for the presence of the omp2 gene of the family Chlamydiaceae, and all isolates were Chlamydophila psittaci. The percentage of diseased goats in which Chlamydiaceae were identified was 16.2%, and all were positive for Cp. psittaci. Of the asymptomatic sheep, 6.7% were positive for the omp2 gene of the family Chlamydiaceae, and again all were positive for Cp. psittaci. In contrast, 42.9% of the samples that were collected from the diseased sheep were positive for Chlamydiaceae, of which 25.7% were Cp. psittaci and 4.8% Cp. pecorum.

Keywords
Chlamydophila pecorum – Chlamydophila psittaci – Egypt – Goat – Restriction fragment length polymorphism polymerase chain reaction (RFLP-PCR) – Sheep.

Introduction
Many farmers in developing countries rear sheep and goats because they are hardy animals that can be produced in resource-poor environments (i.e. areas that lack agricultural land, physical infrastructure, entrepreneurship, commercial finance and technical skills). The family tradition of keeping sheep or goats and the custom of sacrificing sheep for religious festivities may be other important motives for keeping sheep or goats. In addition to the ecological conditions, economic and social conditions also play a part in the choices made by farmers (5, 25). Egypt is a country in which the agro-ecological conditions are believed to have an important impact on the type of small ruminants kept, although both sheep and goats can be found in all agro-ecological zones (7).

Chlamydiaceae are distributed globally and cause acute disease in humans and domestic animals (20, 24). All Chlamyphila species are potential zoonotic pathogens, although Chlamyphila psittaci and Cp. abortus are the most important and best documented (24). Several publications have suggested that the spectrum of disease caused by such chlamydial infections may be much wider than was realised previously (21, 27). A search of the literature on the Chlamydiaceae reveals that there have been complicated changes in chlamydial taxonomy. A revision has occurred on the basis of the phylogenetic relationships deduced from the sequences of the ribosomal RNA (rRNA) cistron, supported by analyses of several other genes, including the gene for the major outer membrane protein and the omp2 gene encoding the 60 kDa cysteine-rich protein, an important component of the bacterial envelope (8, 12, 15). The controversy regarding the nomenclature of Chlamydia and Chlamyphila was discussed by the International Committee on Systematics of Prokaryotes Subcommittee on the Taxonomy of the Chlamydiaceae (15). All members of the subcommittee considered that a single genus, Chlamydia, should be used for species of the genera Chlamydia and Chlamyphila.
sensu. This position is supported by growing evidence. As an example, the percentage of 16S rRNA gene sequence similarity between two members of the genus Chlamydia, Cp. felis and Cp. pneumoniae, is only 95.1%, whereas the similarity is 94.9% between Cp. felis and Chlamydia trachomatis, which were classified in different genera by Everett et al. (12). To close the controversy and to support the proposal of the subcommittee to go back to a single genus, it has been decided to create a working group that will analyse the data derived from all recently available genome sequences of members of the family Chlamydiaceae, including Cp. abortus and Cp. felis (15). This working group will prepare a report that will be discussed by the members of the subcommittee prior to publication. In addition, a new chapter of the second edition of Bergey’s Manual of Systematic Bacteriology that is dedicated to chlamydial taxonomy is in preparation and should be published within the next year.

There are currently four families, six genera and 13 species within the order Chlamydiales that have valid published names (14). They are associated with a wide variety of diseases in domestic animals and humans, including abortion, pneumonia, gastroenteritis, encephalomyelitis, conjunctivitis, arthritis and sexually transmitted diseases (21). The reservoir is large and includes many wild and domestic mammals, but domestic ruminants such as sheep, cattle and goats represent the most frequent source of human infection. Two species of the genus Chlamydophila cause disease in ruminants: Cp. abortus (formerly Chlamydia psittaci serotype 1) and Cp. pecorum (formerly Chlamydia pecorum).

Goats and sheep are widely distributed in urban and rural areas of Egypt. They come into close contact with the human population in parks, gardens and streets and even approach human residences, which raises public health concerns. The presence of sheep and goat faeces in these places has contributed to the spread of infectious agents in the environment. Therefore, the purpose of this study was to examine the faeces of sheep and goats to determine the prevalence of enteric Chlamydiae, which are important zoonotic pathogens that have not been studied previously in Egypt. This was part of a study to characterise the types of Chlamydiaceae found in Egypt and formed part of an investigation of the epidemiology of chlamydiosis in Egypt.

Materials and methods

Specimens

Faecal swabs were collected from sheep (asymptomatic n = 10 and diseased n = 59) and goats (asymptomatic n = 12 and diseased n = 24). The duration of illness, medications given within the last 14 days, and a description of the clinical signs, including the presence or absence of reproductive disease, were requested. Asymptomatic animals were free from diarrhoea and had no history of abortion or upper respiratory disease.

Sampling technique

The faecal samples were taken from asymptomatic animals and from the most severely affected animals with clinical signs of diarrhoea by using a separate disposable glove for each animal. Faecal swabs were suspended in 2 ml of transport medium and were transferred to the laboratory at between 0°C and 4°C (9). The specimens were immediately sent to the Department of Microbiology, Faculty of Veterinary Medicine, Cairo University, where they were processed without delay. The following tests are outlined and recommended by the World Organisation for Animal Health (OIE) (35).

Preparation of inoculum from faecal specimens

For cell culture, sucrose–phosphate–glutamate (SPG [sucrose 74.6 g/l, KH₂PO₄ 0.512 g/l, K₂HPO₄ 1.237 g/l, L-glutamic acid 0.721 g/l]) transport medium was used to prepare a suspension of the faecal specimens. The suspensions were transferred into sterile Eppendorf tubes and centrifuged at 10,000 rpm for 5 min. To eliminate other bacteria and fungi in the faeces, an antibiotic solution containing 10% fetal calf serum, 1 mg/ml vancomycin, 1 mg/ml kanamycin, 1 mg/ml streptomycin, 50 mg/ml gentamicin and 50 mg/ml nystatin was added to the SPG medium, and the samples were left in the refrigerator for 24 h before inoculation (9, 20).

Screening by cell culture

The isolation and cultivation of intracellular agents were performed in Vero cells (23, 35). The cells were originally obtained from the Department of Virology, Faculty of Veterinary Medicine, Cairo University, Egypt. Non-infected cell cultures served as negative controls. Specimens that were positive by phase contrast microscopy underwent further characterisation. Cover slip cultures were infected with the inoculum prepared previously. The cover slips were fixed after an incubation period of 48 h and stained according to the method of Giménez (13).

Inoculation of embryonated eggs

Specific-pathogen-free (SPF) 0-day-old embryonated chicken eggs, acquired from the Poultry Disease Research Institute, Ministry of Agriculture, Egypt, were incubated at 37°C for six days. At the beginning of the sixth day, their vitality was tested with a candling lamp. Seven-day-old embryonated chicken eggs were inoculated with 200 μl
inoculum under sterile conditions, incubated at 37°C, and their vitality was tested on a daily basis. Inoculations were repeated for those eggs whose embryos died in the first three days. Blind passage was performed with embryos that died between days 3 and day 10. The embryos that had not died in ten days were refrigerated on the tenth day, after which blind passage was performed (9, 35). The SPF embryonated chicken eggs that were not inoculated were used as the negative control. At the end of the blind passage procedure, the embryos that had died on days 3–10 were investigated for identification of pathogens. The changes in the yolk sac membrane and embryos were examined. Smears from the egg yolk sac were prepared on sterile slides to be used for Giménez and direct fluorescence-conjugated monoclonal antibody staining and the dry slides for Giménez staining were fixed with methyl alcohol for 5 min and washed with distilled water (32). The dry slides for Giménez staining were fixed with methyl alcohol for 5 min and washed with distilled water (32). The dry slides for direct fluorescence-conjugated monoclonal antibody and immunoperoxidase staining were fixed with cold acetone for 15 min and were left to dry in air. After the slides had dried thoroughly, they were stored at -20°C.

Direct immunofluorescence test

A commercial, fluorescein-labelled, monoclonal antibody conjugate directed against a lipopolysaccharide common to both Cp. psittaci and Chlamydia trachomatis was used according to the manufacturer's instructions (BioMérieux, Lyons, France). Specimens were examined by immunofluorescence microscopy for typical chlamydial elementary bodies.

Immunoperoxidase test

The peroxidase–antiperoxidase method (26) was used. Yolk sac impression smears were washed in 1% hydrogen peroxide (H2O2) in phosphate buffered saline (PBS), followed by 1% skimmed milk in PBS, air-dried, and overlaid with a 1:20 dilution of reference antichlamydial serum (Dienka Sieken Co. Ltd, Tokyo, Japan). After incubation in a humid chamber at 35°C for 30 min the smears were rinsed in 1% skimmed milk in PBS and washed in a stirred bath of PBS for 15 min, dried, overlaid with a soluble peroxidase conjugate (protein A peroxidase [1:1,000], anti-bovine peroxidase-labelled IgG [1:5,000] [Sigma, USA]), re-incubated for 30 min, washed in PBS and dried. Finally, the specimens were immersed for 10 min in a freshly prepared solution of 50 mM Tris-HCl and hydrogen peroxide (0.01% v/v); 3,3 diaminobenzidine tetrahydrochloride (DAB, 0.05% w/v) was then added to flood the smears and left to act for 10 min. This produced a brown insoluble reaction product at the sites of peroxidase activity.

Molecular identification

DNA extraction

Heavily infected yolk sacs were homogenised in 2 ml of PBS (pH 7.3). The suspension was centrifuged at 400 × g for 10 min at 4°C. The Chlamydiae present in the supernatant were washed three times with PBS, then centrifuged at 14,000 × g for 30 min at 4°C. The pellet was directly subjected to DNA extraction using a method described previously (19, 22). The pellet was resuspended in 500 µl TE buffer pH 8.0, and 200 µl of each sample was incubated for 15 min at 55°C with 200 µl of lysis buffer (50 mM Tris-HCl, 20% [wt/vol] sucrose, 20 mM ethylenediaminetetra-acetic acid [EDTA], 0.7% [wt/vol] N-lauroylsarcosine and 200 µg of proteinase K per ml) followed by incubation at 37°C for 45 min with an equal volume of phenol-chloroform (6:4). After centrifugation at 10,000 × g for 30 s, the nucleic acid was precipitated by the addition of 5 M NaCl and ice-cold ethanol. The dried pellet was resuspended in 20 µl diethyl pyrocarbonate-treated water and the supernatant was used in the reaction mixture for amplification.

Polymerase chain reaction conditions

The omp2 polymerase chain reaction (PCR) was performed with the primers Ch1 (5'-ATGTC AAAA CTCATCAGA CGAG-3', positive sense, nucleotides [nt] 529–550) and Ch2 (5'-CCTCTTTAAGGTTTACC-3', negative sense, nt 1093–1115), which amplify a 587-base pair (bp) product from the omp2 gene of Chlamydiaceae family representatives. The reaction was performed according to the method of Hartley et al. (17). All reactions were carried out in a Perkin-Elmer type 480 thermal cycler in 50 µl reaction volumes (17). Reactions contained 1 µl to 10 µl of processed sample, 200 µM each of a mix of deoxynucleoside triphosphates (Amersham-Pharmacia), 5 pmol of each primer, 5 µl of 10 × buffer with 15 mM MgCl2 and 1.25 U of Taq polymerase (which had been used during tests of specificity with non-chlamydia isolates; Roche Biochemicals). Standard amplification conditions for primers Ch1 and Ch2 were 94°C for 4 min (or 95°C for 10 min for Taq Gold) for 1 cycle; 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min for 40 cycles; and a final extension step of 72°C for 7 min for 1 cycle.

Restriction fragment length polymorphism (RFLP) analysis

The method used was described by Hartley et al. (17). Briefly, a discriminatory restriction enzyme was sought by analysis of cutting sites from available sequence data. Restriction with AluI was predicted to give species-specific band lengths, as shown in Table I. Digestion was
performed by incubating a 10 µl aliquot of PCR product with 1 U of enzyme (Promega), 2 µl of 10 × buffer, and 7 µl of water for 1 h at 37°C. The products were analysed by electrophoresis on a 4% Metaphor gel (FMC Bioproducts, Rockland, Maine), stained with ethidium bromide and compared with the predicted analysis.

Table I

<table>
<thead>
<tr>
<th>Species</th>
<th>Fragment lengths (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlamydia trachomatis</td>
<td>158, 119, 114, 84, 77</td>
</tr>
<tr>
<td>Chlamydia muridarum</td>
<td>216, 117, 97, 77, 26, 22, 5</td>
</tr>
<tr>
<td>Chlamydia suis</td>
<td>339, 119, 77, 36, 5</td>
</tr>
<tr>
<td>Chlamydophila abortus</td>
<td>352, 225</td>
</tr>
<tr>
<td>Chlamydophila psittaci</td>
<td>227, 220, 140</td>
</tr>
<tr>
<td>Chlamydophila caviae</td>
<td>355, 140, 105</td>
</tr>
<tr>
<td>Chlamydophila felis</td>
<td>224, 142, 93, 85, 47, 46</td>
</tr>
<tr>
<td>Chlamydophila pneumoniae</td>
<td>444, 127</td>
</tr>
<tr>
<td>Chlamydophila pecorum</td>
<td>397, 193</td>
</tr>
</tbody>
</table>

bp: base pairs

Evaluation of the anti-chlamydial effect of the antibiotics in a monolayer cell-culture assay

The bacterial isolates were divided into aliquots (200 µl of approximately 10⁷ viable Chlamydiae) containing either no antibiotic or one of three antibiotics at the following concentrations: erythromycin (50, 100, 200, 500, 1,000 and 2,000 µg/ml); penicillin G (25, 50, 100, 200, 500 and 1,000 iu/ml) and tetracycline (10, 20, 50, 100, 200, and 500 µg/ml). The mixture was incubated at 35°C for an hour in an attempt to kill the Chlamydiae and tested subsequently for residual viability in a cell culture assay. Confluent Vero cell monolayers were used to isolate viable Chlamydiae after the antibiotic treatments (4).

Results

Growth of Chlamydiae

Overt infection was evidenced by the production of inclusions in culture (Fig. 1).

Detection of Chlamydiae inclusion bodies on impression smears of embryo yolk sac with Giménez stain

Giménez staining was performed for the detection of Chlamydiae in the faecal swabs of asymptomatic and diseased sheep and goats, and 5% of the slides, those in which red inclusion bodies (elementary bodies and reticulate bodies) were seen, were deemed positive (Table II).

Detection of typical chlamydial elementary bodies on impression smears of embryo yolk sac by direct immunofluorescence

Direct fluorescein staining was performed (Fig. 2), and a large number of discrete, densely labelled brown inclusion bodies were seen. Of the slides prepared from the faeces of asymptomatic sheep, 5.5% were positive for Chlamydiae. In addition, 6.5% of the slides from diseased sheep were deemed to be positive (Table II). Of the slides from asymptomatic and diseased goats, 5.6% and 7.5%, respectively, were positive for Chlamydiae (Table II).

Detection of inclusion bodies of Chlamydiae by immunoperoxidase staining of impression smears of embryo yolk sac

After performing immunoperoxidase staining for the detection of Chlamydiae (Fig. 3), 5.6% of the slides prepared from the faeces of asymptomatic sheep were positive for the brown inclusion bodies of Chlamydiae. In addition, 6.7% of the slides from diseased sheep showed a large number of discrete, densely labelled brown inclusion bodies and thus were deemed to be positive (Table II). Of the slides from asymptomatic goats, 5% were positive for the brown inclusion bodies of Chlamydiae. A large number of discrete, densely labelled brown inclusion bodies were also seen on a transparent background in preparations from 5.7% of the faecal swabs collected from diseased goats (Table II).
Polymerase chain reaction identification of Chlamydiae from diseased and asymptomatic goats

Of the faecal samples that were collected from the asymptomatic goats, 50% were positive for the presence of the \textit{omp2} gene of the family Chlamydiaceae (Fig. 4), and all were identified as \textit{Cp. psittaci} (100%) by RFLP-PCR. Of the diseased goats, 16.2% were positive for Chlamydiaceae, and all were also identified by RFLP-PCR to be \textit{Cp. psittaci} (100%) (Table III) (Fig. 5).

Table II
Detection of isolates of Chlamydiaceae using different methods (percentage positive)

<table>
<thead>
<tr>
<th>Test</th>
<th>Asymptomatic Sheep</th>
<th>Diseased Sheep</th>
<th>Asymptomatic Goats</th>
<th>Diseased Goats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vero cell culture</td>
<td>0.0</td>
<td>5.0</td>
<td>0.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Immunofluorescence</td>
<td>5.5</td>
<td>6.5</td>
<td>5.6</td>
<td>7.5</td>
</tr>
<tr>
<td>Immunoperoxidase</td>
<td>5.6</td>
<td>6.7</td>
<td>5.0</td>
<td>5.7</td>
</tr>
<tr>
<td>PCR</td>
<td>6.7</td>
<td>42.9</td>
<td>50</td>
<td>16.2</td>
</tr>
</tbody>
</table>

PCR: polymerase chain reaction

The antibiotics penicillin, tetracycline and erythromycin at different concentrations inactivated the Chlamydiae isolated from different animals.
Discussion

Bacteria in the family Chlamydiaceae are obligate intracellular parasites that infect a diverse array of vertebrates. Chlamydiae cause a wide variety of health problems, including spontaneous abortion in livestock, systemic disease in birds, and both endemic and zoonotic infection of humans (8). However, intestinal infections may be overlooked because they are often asymptomatic in sheep (34). Faecal shedding may be the most important mode of transmission, and the disease syndromes observed reflect the route of infection (6, 28). The syndromes produced vary from localised conjunctivitis, pneumonia, enteritis, encephalitis, polyarthritis or abortion to generalised disease. Only one of these potential clinical manifestations will be overt in a given animal under any particular set of environmental or physiological conditions (28). The organisms can infect most domestic animals, many wild mammals, and more than 100 species of wild and domestic birds are also susceptible. In some cases humans may be infected by contact with diseased animals (24).

The intestinal tract is the natural habitat for Chlamydiae (6, 10, 28, 29, 30). Chlamydial organisms have been isolated from the faeces of sheep (18), and the present report describes the first isolation from the faeces of goats. Inapparent or latent enteric infections are common in most avian species, ruminants (6, 28, 33) and humans (2, 16, 21). In some cases Chlamydiae are excreted by the latently infected host, in other cases the organisms are maintained...
in a non-infectious form. Under circumstances of stress, carrier animals may shed organisms in large numbers or may suffer from clinical disease (28). The research conducted by Bazala and Renda (1) showed that the cause of a number of health problems and diseases could be identified as latent chlamydial infection.

*Chlamydia psittaci* is responsible for a variety of disease syndromes in animals. The organism does not appear to be very host- or tissue-specific and it also has zoonotic potential (2, 9, 16, 20). *Chlamyphila pecorum* is commonly isolated from the digestive tract of ruminants in which the infection is inconspicuous clinically, but the zoonotic impact of this bacterium is still unknown (3). It is also considered that pathogenic strains of *Cp. pecorum* can be spread from the intestines through the blood circulation, as a result of unknown physiopathological events, and may reach the placenta, where they induce abortion. It was not possible in the present study to diagnose mixed chlamydial infections. However, a potential role for Chlamydiae as pathogens of the porcine alimentary tract in mixed infections involving other bacteria has been shown previously (31). Such mixed infections have been found using PCR on samples from field cases (31).

**Conclusions**

As far as the authors are aware, this is the first study of chlamydial infection in goats and sheep with special reference to asymptomatic animals that are latently infected. The effectiveness of the antibiotics penicillin, tetracycline and erythromycin against Chlamydiae isolated in Egypt was also investigated. The impact of infections caused by the family Chlamydiaceae on animal health is difficult to determine, and it is also a public health concern. A possible explanation for the current underestimation of the number of cases of chlamydiosis in animals is that veterinarians and human health professionals are not necessarily aware of the widespread occurrence and zoonotic nature of Chlamydiae. Campaigns to raise awareness, organised by national or local governments, could increase the general degree of attention paid to animal psittacosis. Everyone in contact with birds should be informed about the clinical signs of chlamydial infection and should seek medical attention quickly for themselves and their animals if they have any suspicion of contamination. Humans, especially those who live in rural areas, should be made aware of the risks of zoonotic diseases and how to avoid them (2, 20, 27). Transmission of Chlamydiae by latently infected poultry or other animals should always be suspected in episodes or outbreaks of chlamydiosis in animals, regardless of the presence of clinical signs in the poultry and other animals. This allows detection of animals that are actively shedding the bacteria, which may prevent animal, human and environmental contamination. It is recommended that efficient measures be taken to ensure the high health status of poultry and other animals, and monitoring for Chlamydiae should be conducted on a more regular basis. Poultry are mentioned in particular because of their close association with the human population. In Egypt, between 4 and 7 million families (out of a population of 82 million) raise poultry in their backyards and are at the greatest risk from avian chlamydiosis. This is primarily an avian disease, but because of the way birds are raised in Egypt birds, humans and small ruminants frequently come into close contact.

**Détection de Chlamyphila psittaci et de Chlamyphila pecorum chez des chèvres et des moutons en Égypte**

K.M. Osman, H.A. Ali, J.A. ElJakee & H.M. Galal

**Résumé**

Les auteurs présentent les résultats d’une étude visant à élucider l’épidémiologie de la chlamydiose chez des oivins et des caprins vivant en liberté, aussi bien asymptomatiques qu’atteints de diarrhée. L’isolement des bactéries à partir des échantillons de matières fécales a été réalisé par inoculation en lignée cellulaire Vero ou des embryons de poule. Leur mise en évidence s’est faite par coloration suivant la méthode de Giménez, par immunofluorescence directe à l’aide d’un anticorps monoclonal conjugué à la fluorescéine et par immunoperoxydase. La mise en évidence de l’ADN chlamydien a été réalisée...
Infección de cabras y ovejas por Chlamydophila psittaci y Chlamydophila pecorum en Egipto

K.M. Osman, H.A. Ali, J.A. ElJakee & H.M. Galal

Resumen
Los autores describen un estudio destinado a analizar la epidemiología de la clamidiosis en cabras y ovejas con libertad de movimientos, bien asintomáticas o bien afectadas de diarrea. Para detectar la presencia de clamidias se inocularon muestras fecales en cultivos de células Vero o en embriones de pollo, que después se sometieron a la tinción de Giménez, a detección directa de anticuerpos monoclonales conjugados con fluoresceína y a la prueba de inmunoperoxidasa. Para identificar el ADN clamidial específico se utilizó la amplificación (PCR) del gen omp2 de la familia de las Clamidiáceas. En un 50% de las cabras asintomáticas se observó la presencia de ese gen, que en todos los casos resultó pertenecer a la especie Chlamydophila psittaci. En el caso de las cabras afectadas de diarrea, se detectaron clamidiáceas, siempre pertenecientes a la especie Cp. psittaci, en un 16,2% de ellas. De las ovejas asintomáticas, un 6,7% dio resultado positivo al gen omp2 de la familia Clamidiáceas, de nuevo pertenecientes a Cp. psittaci en todos los casos. En cambio, se detectaron clamidiáceas en un 42,9% de las muestras procedentes de ovejas con diarrea, pero en un 25,7% de los casos se trataba de Cp. psittaci y en un 4,8% de Cp. pecorum.

Palabras clave
Cabra – Chlamydophila pecorum – Chlamydophila psittaci – Egipto – Oveja – Técnica de polimorfismo de longitud de fragmentos de restricción acoplada a la reacción en cadena de la polimerasa (RFLP-PCR).
References


