Epidemiol. Infect. (2000), **125**, 427–439. Printed in the United Kingdom

PCR-based detection of chlamydial infection in swine and subsequent PCR-coupled genotyping of chlamydial *omp1*-gene amplicons by DNA-hybridization, RFLP-analysis, and nucleotide sequence analysis

L. E. HOELZLE[1,3]*, G. STEINHAUSEN[2] AND M. M. WITTENBRINK[3]

[1] *Institute for Medical Microbiology, Infectious and Epidemic Diseases, Ludwig-Maximilians-University Munich, Germany*
[2] *Clinic of Internal Medicine and Surgery of Swine, Ludwig-Maximilians-University Munich, Germany*
[3] *Institute of Veterinary Bacteriology, University of Zurich, Zurich, Switzerland*

(*Accepted 14 May 2000*)

SUMMARY

Lung and intestine of 49 pigs with respiratory diseases and endocervical swabs from 205 sows with reproductive disorders were investigated for chlamydial infection by polymerase chain reaction. PCR primers targeted DNA sequences on the chlamydial *omp1* or *omp2* genes. PCR amplicons were generated from 49·0% of pigs with respiratory disease, from 60·0% of sows with reproductive disorders, from 24·5% of respiratory healthy controls, but from no endocervical swabs from fertile sows. By DNA hybridization, a high prevalence of mixed infections with *Chlamydophila abortus* and *Chlamydia suis* in the porcine lung and intestine was found and confirmed by RFLP and nucleotide analysis. Of the *omp1*-PCR amplicons from endocervical swabs 81·3% were identified as *Chlamydophila abortus*, indicating an association of this chlamydial species with reproductive disorders in sows. Nucleotide sequence analysis of *omp1*-amplicons identified as deriving from *Chlamydia suis* shared a maximum of 82·7% homology with the reference strain S45.

INTRODUCTION

The family *Chlamydiaceae* comprises obligate intracellular eubacteria that are differentiated from other procaryotes by their unique developmental cycle. Both, humans and animals are susceptible for chlamydial infections. Very recently, the family *Chlamydiaceae* is classified into two genera: Genus *Chlamydia* (*C.*) with the species *C. trachomatis*, *C. suis*, and *C. muridarum* and genus *Chlamydophila* (*Chl.*) with the species *Chl. psittaci*, *Chl. pecorum*, *Chl. pneumoniae*, *Chl. abortus*, *Chl. felis*, and *Chl. caviae* [1, 2].

C. trachomatis and *Chl. pneumoniae* are significant human pathogens. Interestingly, *C. trachomatis*-like strains of murine and, more recently, of porcine origin have been described, but these strains are genetically quite different from human *C. trachomatis* and are recently reclassified as the new species *C. muridarum* and *C. suis* in the genus *Chlamydia* [1–8]. The previously described species *Chlamydia psittaci* included genetically and antigenically diverse organisms that infect a wide variety of mammalian and avian hosts and that have been implicated in a range of disease conditions in livestock [9]. By using genetic-based typing systems this heterogenous group of chlamydial strains is reclassified in the new genus *Chlamydophila*. Only the avian-like strains are retained in *Chlamydophila psittaci*, whereas abortion, feline, and guinea-pig strains are transferred to three new species (*Chl. abortus*, *Chl. felis*, *Chl. caviae*, [1, 2]).

In addition, chlamydial organisms are zoonotic causing predominantly a flu-like illness following exposure of humans to birds [7, 10]. Sporadically,

* Author for correspondence: Institut für Veterinärbakteriologie, Universität Zürich, Winterthurerstr. 270, 8057 Zürich, Switzerland.

pregnant women have contracted a gestational chlamydiosis after exposure to ruminant *Chl. abortus* [11, 12]. A group of chlamydial strains from ruminants and swine has been found significantly different from other chlamydial strains previously belonging to the former species *Chlamydia psittaci* and has been, therefore, reclassified as members of the separate species *Chlamydophila pecorum* [1, 2, 13–15].

Pigs are susceptible hosts for *Chlamydiaceae* which cause clinically inapparent infections but are also associated with a wide variety of disease syndromes, including encephalitis, conjunctivitis, enteritis, pneumonia, polyarthritis, or reproductive disorders [16–21]. In swine, the etiologic role of chlamydial species has been further confirmed by experimental reproduction of diseases observed under natural conditions [22–26]. Reports on reclassification of *Chlamydiaceae* from healthy and diseased pigs according to the current taxonomy revealed that pigs are infected by three chlamydial species, namely *Chlamydia suis*, *Chlamydophila pecorum*, and *Chlamydophila abortus* [1–4, 6, 8, 27–29]. Sequence analysis of the *omp1* gene amplified from porcine intestinal tissues showed for the most part homology with *C. suis* and only in a few cases homology with *Chl. abortus* [6]. In specimens from porcine abortion, besides *omp1* gene sequences of *C. suis*, *Chl. pecorum*, and *Chl. abortus* have been detected [27]. In some instances, *Chl. pecorum* was found together with *C. suis*, indicating that pigs are infected simultaneously with more than one chlamydial species.

In conclusion, there are indications that in pigs *C. suis* frequently occurs in the intestine but is also found extraintestinally, whereas *Chl. pecorum* and *Chl. abortus* are detected predominantly from extraintestinal sites including joints, lung or organs of aborted fetuses [6, 27, 28]. The current limited understanding of the relations to disease of the various chlamydial species in swine may be increased by application of molecular methods for detection and subsequent identification to the species level of *Chlamydiaceae* from porcine clinical specimens. PCR amplification and genotypic characterization of e.g. *omp* or ribosomal RNA gene regions of the family *Chlamydiaceae* have been recommended as useful techniques for this purpose [1, 2, 13, 30, 31].

The aim of the present study was to detect and to genotype chlamydial species in clinical specimens from pigs suffering from respiratory disease and reproductive disorders and to compare the prevalence of *Chlamydiaceae* in specimens from diseased and clinically healthy pigs. To detect *Chlamydiaceae* in clinical specimens, cell culture assay and PCR were applied. Interspecies differentiation of *Chlamydiaceae* was performed by nucleic acid-based methods, including restriction fragment length polymorphism (RFLP), Southern blot-, and DNA sequence analysis.

MATERIALS AND METHODS

Samples tested

Between April 1996 and October 1997, a total of 49 pigs of various age, sex, and breed with clinical symptoms of respiratory disease were selected from pigs of the Clinic of Internal Medicine and Surgery of Swine, Veterinary Faculty, LMU-Munich. Specimens from lung and small intestine (ileum) were processed for detection of *Chlamydiaceae* immediately after necropsy. Samples from muscle and kidney of all the animals were collected and stored at −20 °C to monitor for medication of the animals with antibiotics before submission to the clinic. Specimens from lung and small intestine of a total of 49 obviously healthy finishing pigs (90–110 kg body weight on average) were taken aseptically at the Munich abattoir under strict precautions to avoid exogenous contamination. Samples were stored on ice. The interval from sample collection to processing did not exceed 1 h.

Endocervical swabs (cotton-tailed) from 205 sows with symptoms indicative of reproductive disorders (mostly vaginal discharge) from 35 herds and endocervical swabs from 30 clinically healthy fertile sows from five herds were submitted by herd veterinarians for detection of *Chlamydiaceae*. Samples were transported in 0·22 M sucrose-phosphate buffer supplemented with 10% fetal calf serum (FCS) and antibiotics with cold packs and usually arrived within one to two days of collection. Samples were held in the laboratory at 4 °C and processed for chlamydial detection on the day of receipt.

Cell culture assay

Lung tissue and scrapings from the ileal intestinal mucosa were ground as a 1:10 suspension (w/v) in Dulbecco's phosphate-buffered saline (PBS) containing 100 μg streptomycin, 50 μg gentamicin, and 2·5 μg amphotericin B per ml. The suspensions were centrifuged at 500 ***g*** for 10 min at 4 °C to remove cell debris. The supernatant was mixed with an equal volume of PBS plus antibiotics and centrifuged at 2000 ***g*** for

https://doi.org/10.1017/S0950268899004446 Published online by Cambridge University Press

20 min at 4 °C. A 400 μl aliquot from the resultant supernatant was removed for DNA extraction and stored at 4 °C. Another 10 ml from the supernatant were thoroughly mixed with 10 ml PBS plus antibiotics. After centrifugation at 20000 ***g*** for 2 h at 4 °C the pellet was resuspended by ultrasonication in 4 ml of 0·22 M sucrose-phosphate buffer supplemented with 10% FCS and antibiotics in the same concentrations as given above. This suspension was finally centrifuged at 2000 ***g*** for 10 min at 4 °C. Supernatant fluid of the final centrifugate was removed and stored overnight at 4 °C for cell culture inoculation.

Isolation of *Chlamydiaceae* from lung and ileal mucosa was performed by using confluent 24-h-old cell monolayers of Buffalo Green Monkey (BGM) cells (Flow, Meckenheim, Germany) on 12-mm-diameter glass cover slips in shell vials. Initially, 0·5 ml of each specimen was inoculated into each of five shell vials of BGM cells, centrifuged for 1 h at 1000 ***g*** at room temperature, incubated at 37 °C in a humidified atmosphere of 5% CO_2 at 37 °C for 2 h and, after two washings with PBS, given fresh chlamydia isolation medium (Eagle's minimum essential medium supplemented with 5% FCS, 50 μg gentamicin, 2·5 μg amphotericin B, and 2 μg of cycloheximide per ml). Inoculated coverslip cultures were incubated for 10 days with the chlamydia isolation medium being replaced after 7 days. The presence of chlamydial inclusions in cell culture was checked on day 10 by microscopic examination of two coverslip cultures stained by the method of Gimenez [32] and by ascertaining the presence of chlamydial DNA in the culture supernatant by means of PCR. The remaining coverslip cultures were processed for a second passage. Cell cultures were sonicated at 35 W for 1 min on ice. Cell suspensions of each specimen were pooled and used to inoculate four 1-day-old monolayers which were treated as described above. All specimens were serially passed in BGM cells at least three times.

Preparation of genomic DNA for PCR assay

Endocervical swabs were immersed in 500 μl PBS and allowed to stand for about 1 h at room temperature. After a 1 min period of vigorous agitation, the swabs were squeezed and removed. The resulting suspension was centrifuged at 500 ***g*** for 10 min at 4 °C. Aliquots (400 μl) of lung and ileal mucosa homogenates, from washings of endocervical swabs as well as from culture supernatants of BGM cell cultures suspected of chlamydial growth were treated with 45 μl of lysis buffer (100 mM TRIS, 10 mM EDTA, 1 M NaCl, pH 7·4). Sodium dodecyl sulfate (SDS) and proteinase K were added to final concentrations of 1·0 and 0·1 mg/ml, respectively, and the mixture was incubated at 56 °C for 2 h. Samples were incubated at 100 °C for 10 min to inactivate proteinase K. The samples were extracted three times with phenol-chloroform-isoamyl alcohol (25:24:1) according to a standard protocol [33]. Nucleic acids were precipitated by adding 1/10 volume of 3 M sodium acetate and 2 volumes of chilled absolute ethanol. After centrifugation in a microcentrifuge for 30 min at 4 °C , the pellet was rinsed in 70% ethanol, air dried, and resuspended in 50 μl TE buffer (10 mM TRIS, 1 mM EDTA, pH 8·0). For PCR, a 5-μl aliquot was used as a template.

PCR amplification

Oligonucleotides used in this study were synthesized by MWG Biotech, Ebersberg, Germany. Two pairs of oligonucleotide primers which were confirmed to allow amplification of the chlamydial species *C. trachomatis*, *Chl. pecorum*, and *Chl. abortus* were used [13, 34, 35]. Primer pair *omp1* (primer 1: 5′-ATG AAA AAA CTC TTG AAA TCG G-3′; primer 2: 5′-CAA GAT TTT CTA GA(CT) TTC AT(CT) TTG TT-3′) is complementary to an 1050-bp fragment of the *omp1* gene of the investigated chlamydial species (CDS: position 1 to 1043–1061) and amplifies about 93% of the *omp1* gene (GenBank/EMBL accession numbers M17343, M19128, M36703, L39020). Primer pair *omp2* (primer 1: 5′-CAA ACT CAT CAG ACG AG-3′; primer 2: 5′-CCT TCT TTA AGA GGT TTT ACC-3′) amplifies a 590-bp DNA fragment (CDS: position 6 to position 588) of the chlamydial *omp2* gene encoding the cysteine rich 60-kDa protein (accession nos M23001, X55903, X53511, X53512). Moreover, specificity of our PCR was evaluated against a variety of bacterial species, none of them ever gave a positive amplification signal (data not shown).

Both PCR reactions were performed under the same conditions which allowed investigation of samples with both primer pairs at the same time. PCR was performed in 50-μl reaction mixture volumes containing 200 μM concentrations of each primer, 200 μM (each) dATP, dCTP, dGTP, and dTTP; 10 mM Tris/HCl, pH 8·3; 2·5 mM $MgCl_2$; 50 mM KCl, 2·5 U

https://doi.org/10.1017/S0950268899004446 Published online by Cambridge University Press

of Taq DNA polymerase (Boehringer–Mannheim, Germany), and 5 μl of template DNA. The reaction mixture was overlaid with 20 μl mineral oil and subjected to 30 cycles of amplification in a DNA thermal cycler (Perkin–Elmer–Cetus). Each cycle consisted of a 1 min denaturation step at 94 °C, a 1 min annealing step at 55 °C, and a 2 min extension step at 72 °C. The final cycle included a 7 min extension step at 72 °C to ensure full extension of the amplification product. DNA preparations from the *C. suis* strain PCLH296, *Chl. pecorum* strain LW613, and the ovine *Chl. abortus* strain OCLH196 were used as positive controls. Two control tubes containing distilled water and BGM cell DNA in place of template DNA were included in each batch of amplifications. All batches of DNA extracts from porcine specimens (tissue, endocervical swabs) were spiked with 10 fg of positive control DNA to detect PCR inhibition. A 10-μl volume of the PCR amplified reaction mixture was analysed by electrophoresis on a 1% agarose gel (Agarose NEEO, Roth, Karlsruhe, Germany) containing ethidium bromide in the presence of a 100-bp and a 1-kb DNA ladder (Invitrogen, Netherlands). Molecular sizes of the amplification products relative to those of the 1-kb and the 100-bp DNA ladder were assessed by a computer-aided bio-image system (BioProfil 3·1, LTF, Wasserburg, Germany).

Southern blot analysis of *omp1*-PCR products

Amplification products of the *omp1*-PCR were further analysed by Southern blot hybridization using three different DNA probes. DNA probe *Cab* was derived from the ovine *Chl. abortus* strain OCLH196 (GenBank accession no. AJ004873), probe *Csu* from the porcine, *C. suis* strain PCLH296 (GenBank accession no. AJ004880), and *Cpec* from the *Chl. pecorum* strain LW613 [13]. All DNA probes consisted of the corresponding *omp1*-PCR amplification product which was randomly labelled with DIG-dUTP according to the manufacturer's instructions (Boehringer–Mannheim, Germany). Specificity and cross-reactivity of the DNA probes *Cab*, *Cpec* and *Csu* were confirmed by a series of proceeding control studies demonstrating that the three probes used show neither cross reaction nor hybridize with DNA purified from other bacteria or viruses from swine, e.g. mycoplasma (data not shown).

Hybridization was performed according to standard protocols [33]. Briefly, PCR amplification products were analysed on a 1% agarose gel and transferred to Hybond-N nylon membranes by capillary transfer with 1·5 M NaCl, 0·25 M NaOH. The hybridization buffer consisted of 0·75 M NaCl-75 mM Na-citrate (pH 7·0; 5 × SSC) containing 0·02% (w/v) sodium dodecyl sulphate (SDS), 0·1% N-lauroyl-sarcosine (sodium salt), 5% (w/v) blocking agent (Boehringer–Mannheim, Germany), and 50% (v/v) formamide. The hybridization reaction was performed overnight at 42 °C. Thereafter, blots were washed twice for 5 min in 2 × SSC-0·1% SDS at ambient temperature followed by two subsequent washings in 0·1 × SSC-0·1% SDS at 64 °C for 20 min. Hybridized probes were detected by using alkaline phosphatase conjugated anti-DIG antibody and the color reagents provided in the DIG labelling and detection kit (Boehringer–Mannheim, Germany).

RFLP analysis

Amplification products from the *omp1*-PCR were purified by using the QIAquick purification kit (Qiagen, Hilden, Germany). Restriction endonuclease *Alu*I (Boehringer–Mannheim, Germany) was used to cleave PCR products according to the manufacturer's recommendations. 40 μl of each *omp1*-PCR product was cleaved with 10 units *AluI* at 37 °C overnight. Following digestion, the DNA fragments were precipitated with 3 M Na-acetate and absolute ethanol and redissolved in 10 μl TE buffer. Restriction digests (10 μl) were separated electrophoretically in a 3·0% agarose gel (Small DNA agarose, Biozym, Oldendorf, Germany). Gels were stained with 0·1% ethidium bromide for 15 min and photographed under UV illumination. The bio-image system was used to assess fragment sizes of the restriction patterns against a 100 bp DNA ladder (Invitrogen, Netherlands).

DNA sequencing

Prior to DNA sequencing, amplification products from the *omp1*-PCR were cloned into the plasmid vector pCR2.1 (Invitrogen, Netherlands) and plasmid DNA of each clone was purified with the Qiagen Plasmid Kit (Qiagen, Hilden, Germany). For each sequenced *omp1* amplification product two different plasmid clones from two independent cloning reactions were selected.

Sequencing was performed by MWG Biotech (Ebersberg, Germany) according to the dideoxy method described by Sanger and colleagues [36].

Nucleotide sequences were analysed and aligned to each other by using HUSAR (Heidelberg Unix Sequence Analysis Resources). Nucleotide and deduced amino acid sequences were compared with data bank entries of other *Chlamydiaceae* strains accessioned in the EMBL and GenBank database.

Nucleotide sequence accession numbers

Accession numbers for the DNA regions submitted to the EMBL nucleotide sequence database are from AJ004873 to AJ004880 and from AJ005613 to AJ005618.

Statistical analysis

The detection rate of *Chlamydiaceae* from porcine specimens for the different groups of pigs examined were compared by using the χ^2 test. P values of $\leqslant 0{\cdot}05$ were considered significant.

Assay for antibiotic residues in porcine tissues

To monitor pigs for pretreatment with antibiotics effective against *Chlamydiaceae*, a screening assay for the microbiological detection of antibiotic residues in kidney and muscle tissues of slaughter animals was performed according to German meat hygiene laws [37]. The test is based on growth inhibition of *Bacillus subtilis BGA* (German Collection of Microorganisms, Braunschweig, Germany, DSM-No. 618) which is the official test strain for the detection of antibiotic residues in meat. Briefly, cylindrical samples of 2 mm height and 8 mm in diameter cut out from kidney and muscle tissues were placed on standardized agar medium, pH 6·0 containing approximately 10^4 spores of *B. subtilis*. After 24 h incubation at 30 °C, plates were examined for zones of growth inhibition around the tissue samples. Filter paper disks containing 10 μg tetracycline were applied to agar plates as a positive control in every run (Unipath, Wesel, Germany). Inhibition zones of $\geqslant 2$ mm in diameter were considered indicative for the presence of antibiotic residues in tissue specimens.

RESULTS

Detection of *Chlamydiaceae* in porcine specimens

PCR amplifications carried out with *omp1* or *omp2* primers and DNA extracted from porcine *C. trachomatis*-like strain PCLH296, *Chl. pecorum* strain LW613, and *Chl. abortus* strain OCLH196 gave rise to the expected 1-kb- or 590-bp fragment, respectively. DNA-fragments clearly detectable on the gel were amplified from 1 fg of *Chl. abortus* template DNA in the PCR reaction mixture, equivalent to 100–200 genome copies. PCR amplifications carried out on DNA extracts from all batches of porcine specimens spiked with chlamydial positive control DNA showed no evidence of amplification inhibition. The results of PCR-based detection of *Chlamydiaceae* in porcine lungs, intestines, and endocervical swabs are summarized in Table 1. In 24 of 49 pigs diagnosed with bronchopneumonia (49·0%), *omp1*-PCR amplification of DNA extracts from lung and intestine resulted in a 1-kb band when analysed by gel electrophoresis. Parallel PCR reactions using the *omp2* primers amplifying a 590-bp fragment on the chlamydial *omp2* gene revealed concordant positive and negative results in all cases. Representative examples of DNA-fragments amplified from porcine specimens by *omp1*-PCR and *omp2*-PCR are shown in Figure 1. In lung and intestine from 49 obviously healthy slaughter pigs, chlamydial DNA fragments were amplified by *omp1*-PCR and *omp2*-PCR in 12 cases (24·5%). The detection rate of chlamydial DNA was significantly higher in pigs with bronchopneumonia than in slaughter pigs ($P < 0{\cdot}05$, χ^2 test; Table 1).

In the group of 49 pigs with bronchopneumonia, chlamydial organisms were isolated by means of cell culture from seven animals (14·3%). In five cases, *Chlamydiaceae* were only grown from the intestine, in a further case from lung and intestine. One isolate was obtained from a pneumonic lung. In the group of 49 obviously healthy slaughter pigs, *Chlamydiaceae* were isolated from the intestine of one animal (2·0%). Isolation of *Chlamydiaceae* in BGM cell culture was successful only from PCR positive specimens. In BGM cells, appearance of intracellular chlamydial inclusions took at least 10 days but the majority of chlamydial strains required more than three passages before visible inclusions were detected in any number.

A total of 205 endocervical swabs from sows diagnosed with reproductive disorders associated with vaginal discharge were available for PCR. DNA fragments of the expected sizes were amplified by *omp1*- and *omp2*-PCR from 123 extracted endocervical swabs (60·0%). *Chlamydiaceae* were not detected in any of the 30 endocervical swabs from healthy fertile sows by means of PCR. The rate of

https://doi.org/10.1017/S0950268899004446 Published online by Cambridge University Press

Table 1. *PCR results for specimens from diseased pigs and healthy control pigs*

Origin of positive PCR product	No. of animals tested/no. positive (%)	
	Pigs with respiratory disease	Healthy slaughter pigs
Lung*	49/2 (4·1)	49/0 (0)
Intestine*	49/13 (26·5)	49/6 (12·2)
Lung and intestine†	49/9 (18·4)	49/6 (12·2)
Total positive	49/24 (49·0)‡	49/12 (24·5)‡
Origin of positive PCR product	Sows with reproductive disorders	Healthy fertile sows
Endocervical swabs	205/123 (60·0)§	30/0 (0)§

* In these cases amplification products were found only in lung or only in intestine.
† In these case amplification products were found in both lung and intestine.
‡ $P < 0{\cdot}05$.
§ $P < 0{\cdot}001$; χ^2 test.

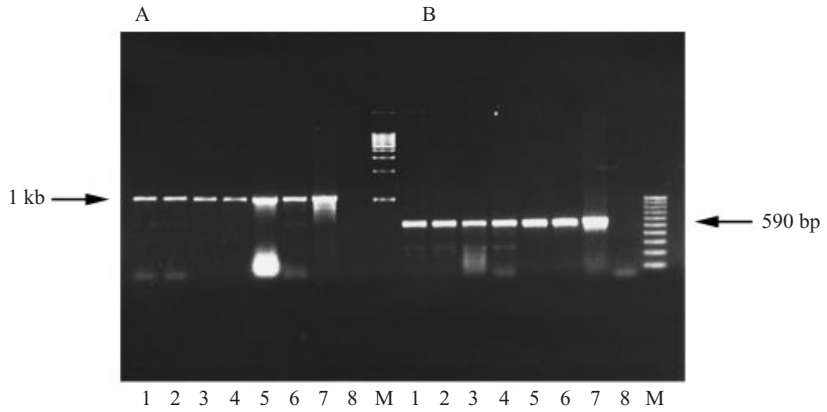

Fig. 1. Ethidium bromide-stained 1% agarose gel of PCR-amplified products obtained with primers specific for chlamydial *omp1* (*omp1*-PCR) and *omp2* (*omp2*-PCR) gene sequences. (A) *omp1*-PCR Lanes: 1–4, clinical porcine specimens; 5–7, positive controls (DNA from *Chl. abortus* strain OCLH196 (5), from *C. suis* strain PCLH296 (6), from *Chl. pecorum* strain LW613 (7); 8, no template DNA (negative control); M, molecular size standard (1 kb ladder). (B) *omp2*-PCR Lanes: 1–4, clinical porcine specimens; 5–7, positive controls; 8, negative control; M, molecular size standard (100 bp ladder).

PCR positivity was significantly higher among specimens from sows with reproductive disorders than among specimens from healthy fertile sows ($P < 0{\cdot}001$, χ^2 test; Table 1).

Southern blot analysis of *omp1*-PCR products

A representative Southern blot is shown in Figure 2 and the different hybridization reactivities of a total of 126 1-kb *omp1*-PCR amplicons from porcine specimens (lung, intestine, endocervical swabs) are summarized in Table 2. All 18 amplicons from lung and intestine of healthy slaughter pigs hybridized specifically with both, the DNA probe specific for *Chl. abortus* (*Cab*) and with the probe specific for porcine *C. trachomatis*-like organisms (*Csu*, recently reclassified *C. suis*, [1, 2]), but not with the DNA probe *Cpec* specific for *Chl. pecorum*. On testing of 33 amplicons from lung and intestine of pigs with a history of respiratory disease, 18 amplicons (54·5%) hybridized specifically with both *Cab* and *Csu*. The remaining 15 *omp1*-PCR amplicons hybridized only with *Cab* ($n = 6$, 18·2%) or with *Csu* ($n = 9$, 27·3%), i.e. amplicons that gave a positive hybridization signal with *Cab* did not hybridize with *Csu* and vice versa. In no case was a specific reaction with the tested amplicons with *Cpec* observed.

Irrespective of the clinical history, *omp1*-PCR amplicons from porcine lung and intestine were confirmed by Southern blot analysis to originate from

https://doi.org/10.1017/S0950268899004446 Published online by Cambridge University Press

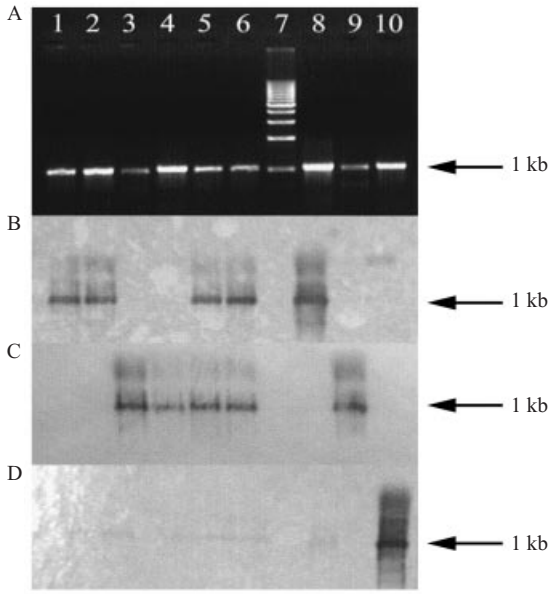

Fig. 2. Southern hybridization of *omp1*-PCR-amplified products (A) with the DIG-labelled *Chl. abortus*-specific DNA probe *Cab* (B), the DIG-labelled *C. suis*-specific DNA probe *Csu* (C), and the DIG-labelled *Chl. pecorum*-specific DNA probe *Cpec* (D). (A) lanes: 1–6, ethidium bromide-stained 1% agarose gel showing 1050-bp *omp1*-PCR products from porcine clinical specimens (lane 1, pm234; 2, pm364; 3, pmpclh; 4, pm39; 5, pmsh47; 6, pmd623); 7 molecular size standard (1 kb ladder); 8–10, *omp1*-PCR from *Chl. abortus*, *C. suis*, and *Chl. pecorum* (positive controls). (B) lanes 1–10, specific binding of the probe *Cab* with *omp1*-PCR products in lane 1, 2, 5, 6 and 8 (positive control). (C) lanes 1–10, specific binding of the probe *Csu* with *omp1*-PCR products in lane 3–6, 9 (positive control). (D) lanes 1–10, specific binding of the probe *Cpec* with the *omp1*-PCR product in lane 10 (positive control).

Chl. abortus in 18·2%. *C. suis* was identified as the origin of 27·3% of amplicons from lung and intestine. Specific hybridization signals with both DNA probes indicating mixed infections with two chlamydial species were obtained in 100% of amplicons from healthy pigs and in 54·5% of amplicons from pigs with respiratory disease. In contrast, out of 75 *omp1*-PCR amplicons from cervical swabs 61 (81·3%) reacted only with the DNA probe *Cab*. Notably, no amplicon from the cervix hybridized exclusively with *Csu*. A group of 14 amplicons (18·7%) yielded specific hybridization signals with both probes. In no case a reactivity with the DNA probe *Cpec* was detected. The rate of Southern blot reactivity with *Cab* was significantly higher and with *Csu* significantly lower in the group of *omp1*-PCR amplicons from the cervix of sows with reproductive disorders compared with amplicons from lung and intestine ($P < 0{\cdot}001$, χ^2 test, Table 2).

In order to classify chlamydial isolates grown from porcine species in BGM cell cultures, DNA purified from chlamydial particles from cell culture supernatants were subjected to *omp1*-PCR and subsequent Southern blot analysis. Out of six *omp1*-PCR products amplified from intestinal isolates, four were reactive with both probes whereas two amplicons hybridized only with one DNA probe, *Cab* or *Csu*. One isolate, grown in parallel from intestine and lung of a pig suffering from bronchopneumonia, was reactive exclusively with the *Csu* probe. One isolate delivered from a pneumonic lung gave a specific signal with the *Csu* probe.

RFLP-analysis of *omp1*-PCR products

After digestion of 20 *omp1*-PCR amplicons from porcine specimens (lung, intestine, endocervical swabs) with *Alu*I, three distinct RFLP patterns designated I-III were obtained. The reproducibility of banding patterns was 100% for six triplicate analyses on the gel. Exact fragment sizes were determined by DNA sequence analysis of selected *omp1*-PCR amplicons. Representative examples of RFLP patterns are shown in Figure 3. Differentiation by RFLP analysis correlated well with the Southern blot results. Amplicons that hybridized only with probe *Cab* demonstrated two *Alu*I restriction patterns designated as Ia and Ib that were clearly distinct on the gel. Pattern Ia consisted of five visible fragments ranging in size from 60 to 195 bp (60, 80, 160, 170, 195 bp), pattern Ib yielded four fragments ranging in size from 40 to 170 bp (40, 80, 140, 170 bp). In comparison, *omp1*-PCR amplicons only reactive with the DNA probe *Csu* produced two clearly distinct *Alu*I restriction patterns IIa and IIb. The vast majority yielded pattern IIa, consisting of six fragments from 60 to 250 bp (60, 110, 130, 140, 170, 250 bp). Only one amplicon showed pattern IIb with six fragments from 40 to 260 bp (40, 80, 130, 180, 210, 260 bp). The *Alu*I cleavage of PCR amplicons that hybridized with both DNA probes in equal intensity resulted in an unique pattern III consisting of eight fragments ranging in size from nearly 60 to 250 bp. DNA fragments were nearly in the same molecular size range as fragments of *Alu*I patterns I and II indicating that the *Alu*I pattern III was composed of RFLP patterns from hybridization group I and II. *Alu*I-digestion of an *omp1*-amplification product derived from *Chl. pecorum* used as a control revealed four fragments

https://doi.org/10.1017/S0950268899004446 Published online by Cambridge University Press

Table 2. *Analysis of* omp1*-PCR amplification products from porcine specimens*

Origin of *omp1*-PCR amplification product	No. of amplicons tested/no. positive (%) Reactivity with DNA proves		
	Probe *Cab*†	Probe *Csu**	Probe *Cab* and probe *Csu*
Healthy slaughter pigs			
Lung	0	0	0
Intestine	6/0 (0)	6/0 (0)	6/6 (100·0)
Lung and intestine	12‡/0 (0)	12/0 (0)	12/12 (100·0)
Total positive	18/0 (0)	18/0 (0)	18/18 (100·0)
Pigs with respiratory disease			
Lung	2/0 (0)	2/1 (50·0)	2/1 (50·0)
Intestine	13/2 (15·4)	13/3 (23·1)	13/8 (61·5)
Lung and intestine	18§/4 (22·2)	18/5 (27·8)	18/9 (50·0)
Lung	9/2 (22·2)	9/2 (22·2)	9/5 (55·6)
Intestine	9/2 (22·2)	9/3 (33·3)	9/4 (44·4)
Total positive	33/6 (18·2)	33/9 (27·3)	33/18 (54·5)
Sows with reproductive disorders endocervical swabs	75/61 (81·3)	75/0 (0)	75/14 (18·7)

* *Chlamydophila abortus* specific DNA probe
† *Chlamydia suis* specific DNA probe
‡ These tested *omp1*-PCR products were delivered from amplicons of six lung and intestine infected pigs from slaughter.
§ These tested *omp1*-PCR products were delivered from amplicons of nine lung and intestine infected pigs with respiratory diseases.

ranging from 130 to 300 bp (130, 180, 250, 300 bp). This restriction pattern was found in no case when *omp1*-PCR products from porcine specimens were digested. In addition, RFLP analysis of *omp1*-PCR amplicons from porcine chlamydial isolates grown in BGM cell cultures also correlated with the identification by Southern blot analysis.

DNA sequence analysis

The nucleotide and deduced amino acid sequences of three amplicons reacting in the hybridization group I (pm234, pm326, pm364) showed an overall sequence homology of about 99%. All three sequences fit at best with the *omp1* gene sequence of the ovine abortion type strain B577 of *Chl. abortus* (98·0%). When comparing the three representative amplicons of group II (pm39, pm197, pmpclh) an overall nucleotide sequence homology of 88·3% was observed within the group. Amino acid homology was about 92·0%. Comparison of the three sequences with data bank entries revealed the best correspondence with the *omp1* gene sequences of *C. trachomatis* (81·1–82·7%). Alignment of DNA sequences from the three porcine *C. trachomatis*-like major outer membrane protein (MOMP) alleles with the sequences of the known *C. suis* prototype strain S45 revealed that the hybridization group II consists of at least two new *omp1* alleles of *C. suis* (Fig. 4). These novel alleles had deduced peptide sequences which were about 20% divergent from *C. suis* S45. Most differences were clustered in the region of the variable segments (VS) I to IV of the MOMP protein of *C. suis* S45 (Fig. 4). Out of seven amplification products from hybridization group III (reactive with *Cab* and *Csu*) DNA sequences of three amplicons (pmd1340, pmsh47, pm220) were nearly 81% homologous to *C. trachomatis omp1* gene sequences from data bank entries. Alignment of these sequences with the *C. suis* S45 strain revealed an amino acid homology of about 79%. DNA sequences pmd1340, pmsh47, pm220 were identical with corresponding sequences pm197/pmpclh of the hybridization group II representing *C. suis*. The remaining four amplicons (pmd623, pm225, pm112, pmsh1) were identified as *Chl. abortus* with a DNA sequence homology rate of 99·0%. Thus, the detection of PCR amplification products reactive with

https://doi.org/10.1017/S0950268899004446 Published online by Cambridge University Press

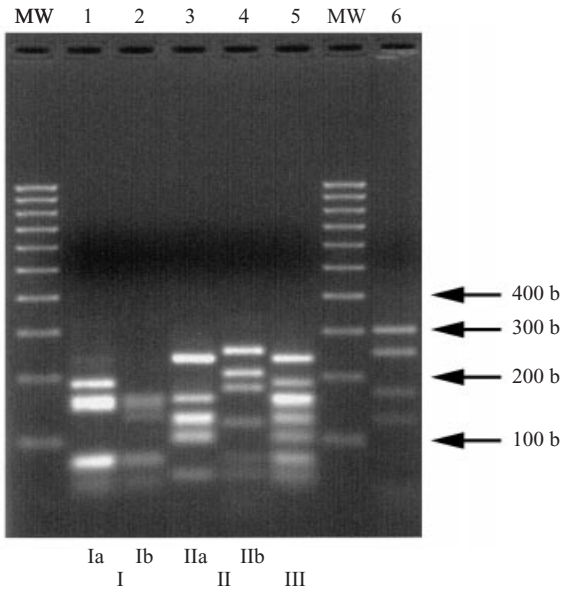

Fig. 3. RFLP-analysis with *Alu*I of *omp1*-PCR-amplified products of clinical porcine specimens representing amplicons from hybridization group 1 (only reaction with DNA-probe *Cab*), II (only reaction with DNA-probe *Csu*) and III (reaction with both *Cab* and *Csu*). Lanes: 1, *Alu*I-pattern Ia from amplicon pm364; 2, pattern Ib from amplicon pm234; 3, pattern IIa from amplicon pmpclh; 4, pattern IIb from amplicon pm39; 5, pattern III from amplicon pmsh47; 6, *Alu*I-pattern from *Chl. pecorum* strain LW613; MW, molecular size standard (100 bp ladder).

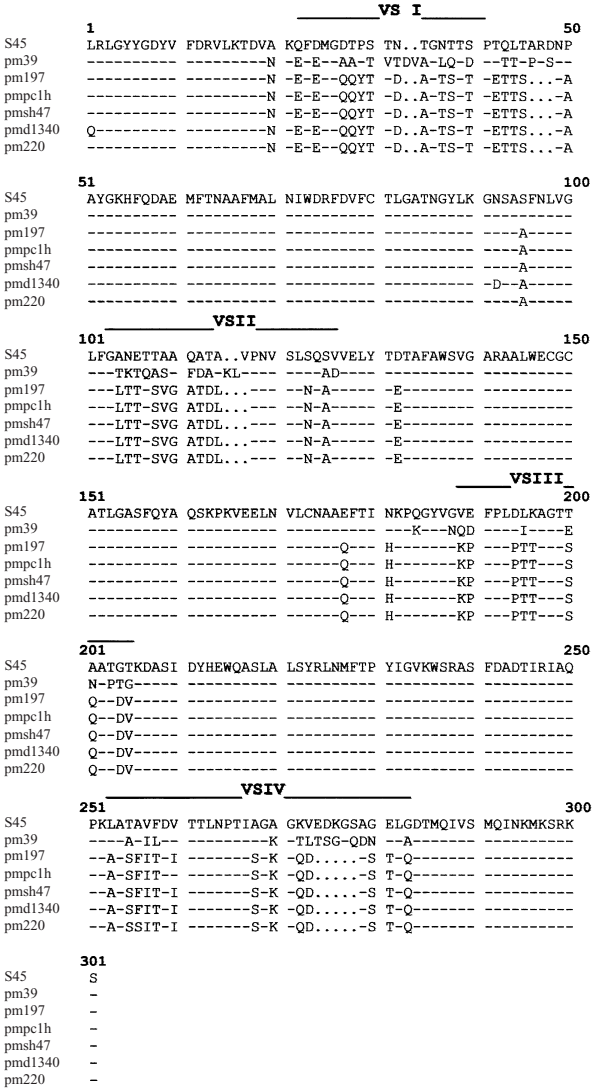

Fig. 4. Comparison of deduced amino acid sequences of variable segments (VS) I-IV of porcine *C. trachomatis*-like *omp1*-PCR products with prototype strain of *C. suis* S45. Two novel alleles of porcine *C. suis omp1*-gene are found represented by pm39 (lane 2) and pm197 to pm220 (lanes 3–7). Hyphens indicate identical amino acids, and dots represent missing amino acids.

both DNA probes is strongly indicative for mixed infections with *Chl. abortus* and *C. suis*.

After cloning of amplicons of hybridization group III, *E. coli* clones containing *omp1* gene sequences of either *Chl. abortus* or *C. suis* were obtained as was confirmed by DNA-DNA hybridization. Furthermore, DNA sequences of those amplicons cloned in *E. coli* were nearly identical with *omp1* gene sequences either of *Chl. abortus* or *C. suis*.

Antibiotic residues in porcine tissues

Kidney and muscle specimens from 7 out of 17 animals (41·2%) for which cell culture failed to detect *Chlamydiaceae* in the intestine or lung but which gave a positive PCR amplification reaction exhibited an inhibition zone of $\geqslant 2{\cdot}0$ mm. In seven pigs with concordant chlamydia positive cell culture and PCR, an equivalent inhibition zone of $\geqslant 2{\cdot}0$ mm was detected in specimens from one animal (14·3%). The rate of antibiotic detection was not significantly different between those groups ($P > 0{\cdot}05$, χ^2 test).

DISCUSSION

For the detection of *Chlamydiaceae* in pigs, PCR was compared with cell culture. Cell culture isolation of chlamydial organisms is considered to be a rather sensitive method. However, this technique is time-consuming and therefore not as easy to perform for large-scale epidemiological surveys as the methods described here [10, 38, 39]. Mainly due to insufficient storage conditions and prolonged transport at am-

https://doi.org/10.1017/S0950268899004446 Published online by Cambridge University Press

bient temperature of clinical specimens submitted by veterinarians, false negative chlamydia cell culture was more likely to occur than false positive PCR. Furthermore, the amount of viable chlamydial organisms in clinical specimens from diseased pigs was probably reduced by antibiotic treatment e.g. with tetracyclines. By applying a bioassay, antibiotic residues were more often detected in the samples of pigs categorized PCR positive/cell culture negative (41·2%) than in the samples of pigs categorized PCR positive/cell culture positive (14·3%). Although this difference could not be confirmed statistically because of the low number of cases included in our sample, routine antibiotic treatment of diseased animals is a probable explanation for the low detection rate of *Chlamydiaceae* by cell culture. Consequently, in addition to 12 PCR positive porcine specimens from which chlamydial organisms could be recovered in culture, 37·8% of cell-culture negative specimens were PCR positive. Although false positive PCR results due to contamination with natural chlamydial DNA can never be excluded, the probability of accidental contamination was minimized by strict precautions like negative control measures in every PCR run and strict separation of PCR steps. Positivity of negative PCR controls or clustering of positive PCR amplification products yielding identical RFLP- or hybridization patterns were not observed. Thus, PCR is considered an effective tool in routine diagnosis of chlamydial infections in swine. However, cell culture is essential despite the difficulty in growing *Chlamydiaceae* from porcine specimens, because it provides a source of new strains.

In our survey, chlamydial DNA was amplified from 49·0% of pigs suffering from bronchopneumonia. Positive PCR signals were obtained either from lung or intestine or from both locations. The prevalence of *Chlamydiaceae* in pneumonic pigs was significantly higher than in healthy slaughter pigs. As earlier field studies, our data are also indicative for an aetiological role of *Chlamydiaceae* in porcine respiratory disease [40, 41]. The significance of *Chlamydiaceae* in porcine pneumonia has been confirmed by experimental inoculation of swines with chlamydial isolates of different origin [22, 23, 25, 42].

In the present study, DNA–DNA hybridization with specific probes allowed us to assign *omp1* amplification products from the porcine intestine and lung to the species *Chl. abortus* and *C. suis*. These findings were confirmed by RFLP- and DNA sequence analysis of *omp1* amplification products. According to these methods, mixed infections with *Chl. abortus* and *C. suis* were found in 70·6% of intestinal specimens. The predominance of mixed infections with both chlamydial species suggests that the porcine intestinal tract is a common reservoir for two chlamydial species which probably lead to respiratory infection. Our findings corroborate those of other studies which have also demonstrated chlamydial mixed infections in the porcine intestinal tract [6]. Interestingly, in no case an infection with *Chl. pecorum* was found. Aside from the significantly higher prevalence of *Chlamydiaceae* in pneumonic animals no evident association between the presence of respiratory disease and one particular chlamydial species was seen in our study.

In endocervical swabs from sows with reproductive disorders, the prevalence of *Chlamydiaceae* was unambiguously higher than in specimens from clinically healthy fertile sows. These results are strongly suggestive for an etiological role of *Chlamydiaceae* in endometritis associated with a high rate of return to estrus since other common infectious causes of porcine endometritis have been ruled out before by means of standard microbiological examinations. In fact, the significance of our results is further supported by recent seroepidemiologic studies reporting a relationship between prevalence of chlamydial infection and reproductive failure in sows [43]. Moreover, strong evidence for the pathogenic role of *Chlamydiaceae* in the porcine genital tract has emerged from experimental chlamydial infections [26, 44]. In contrast to the predominance of mixed infections with *Chl. abortus* and *C. suis* in the porcine respiratory and intestinal tract, most of amplification products from the porcine cervix were identified as *Chl. abortus*. Mixed infections with both *Chl. abortus* and *C. suis* were detected in only 18·7% of cases; single infections with *C. suis* or with *Chl. pecorum* were not found. Thus, evidence is provided of a strong association between infection of the upper genital tract of sows with *Chl. abortus* and clinical disease characterized by abortion, endometritis, cervical discharge, and increased return to estrus. The finding of coinfection with *C. suis* in 18·7% of sows with cervical *Chl. abortus*-infection could be explained by the probable route of infection. This paper has confirmed that the porcine intestine is commonly infected with both chlamydial species which are shed in the feces [40].

Results from a recent serological survey support the view that poor hygiene is indeed a predisposing factor

for spreading chlamydial infection in swine herds [43]. Thus, clinically manifest genital chlamydiosis in sows may probably result from ascending infections along the faecal-genital route with genitopathogenic *Chl. abortus* accompanied in a number of cases by *C. suis* with a presumably limited genitopathogenicity. This is consistent with the findings of a previous study in gnotobiotic piglets in which a representative strain of *C. suis* exhibited marked intestinal pathogenicity whereas the ovine strain of *Chl. abortus* appeared to be apathogenic in the porcine intestine [45]. Hitherto, the relations to disease of various chlamydial species recovered from pigs were not well understood. Moreover, the number of porcine chlamydial isolates from clinical cases is too low to decide whether all species have the same pathogenicity. To this end, the finding of mixed infections with *C. suis* and *Chl. pecorum* in porcine abortions by Schiller and co-workers [27] does also not conflict with our explanation that *Chl. abortus* is clearly more associated with reproductive disorders in sows due to its marked genitopathogenicity [27]. The etiologic role of *Chl. abortus* as a significant cause of reproductive disorders, including abortion, endometritis or increased return to estrus in cattle, goats, and sheep, is well documented but remains to be established by experimental studies in the pig [9, 46–49].

PCR-based genotyping by RFLP-analysis of the chlamydial *omp1* gene showed a high discriminatory power making this method a worthwhile tool in epidemiological studies of chlamydiosis in lifestock including swine [13, 30, 50–53]. In our studies, two different *Alu*I-RFLP patterns were found for PCR amplification products identified as *Chl. abortus* by Southern blot hybridization. Results for this RFLP-analysis were confirmed definitively by nucleotide sequencing, indicating that porcine *Chl. abortus* and *C. suis* can be further differentiated into genotypes. Moreover, by nucleotide sequencing two novel *omp1* alleles of *C. suis* were identified in addition to other well described *C. suis omp1* alleles [5, 30]. Nucleotide sequences of our novel *omp1* alleles were clearly different from corresponding sequences of the *C. suis* reference strain S45. Mutations were clustered in the variable segments (VS) I-IV of the *omp1* gene locus. Additionally, a few mutations were observed in the conserved regions of the *omp1* gene. While this work was in progress, a paper providing evidence for existence of numerous variants of *omp1* alleles of *C. suis* was published by Kaltenboeck and colleagues [5]. Our results are in good agreement with the data of Kaltenboeck and colleagues, who have described novel alleles which were 11·6–19·0% divergent from *C. suis* reference strain S45. VS I, II, and IV of the chlamydial *omp1* gene encode surface-exposed peptides representing species-, subspecies-, and serovar-specific epitopes. The high frequency of mutations in VS IV imply that different serovariants of *C. suis* may exist.

In conclusion, PCR allows the specific and sensitive detection of *Chlamydiaceae* from porcine specimens. PCR-coupled RFLP analysis showed a high discriminatory power to differentiate chlamydial PCR amplification products to the species and subspecies level. Its use allowed to demonstrate a strong association between genital infection with *Chl. abortus* and reproductive disorders in sows.

REFERENCES

1. Everett DE, Bush RM, Andersen AA. Emended description of the order *Chlamydiales*, proposal of *Parachlamydiaceae* fam. nov. and *Simkaniaceae* fam. nov., each containing one monotypic genus, revised taxonomy of the family *Chlamydiaceae*, including a new genus and five new species, and standards for the identification of organisms. Int J Syst Bacteriol 1999; **49**: 415–40.
2. Everett DE, Andersen AA. Identification of nine species of the *Chlamydiaceae* using PCR-RFLP. Int J Syst Bacteriol 1999; **49**: 803–13.
3. Kaltenboeck B, Storz J. Biological properties and genetic analysis of the *ompA* locus in chlamydiae isolated from swine. Am J Vet Res 1992; **53**: 1482–7.
4. Kaltenboeck B, Kousoulas KG, Storz J. Structures of and allelic diversity and relationships among the major outer membrane protein (*ompA*) genes of the four chlamydial species. J Bacteriol 1993; **175**: 487–502.
5. Kaltenboeck B, Schmeer N, Schneider R. Evidence for numerous *omp1* alleles of porcine *Chlamydia trachomatis* and novel chlamydial species obtained by PCR. J Clin Microbiol 1997; **35**: 1835–41.
6. Schiller I, Koesters R, Weilenmann R, Kaltenboeck B, Pospischil A. PCR detection of porcine *Chlamydia trachomatis* and ruminant *Chlamydia psittaci* serovar 1 DNA in formalin-fixed intestinal specimens from swine. J Vet Med B 1997; **44**: 185–91.
7. Storz J, Kaltenboeck B. Diversity of chlamydia-induced disease. In: Woldehiwet Z, Ristic M, eds. Rickettsial and chlamydial diseases of domestic animals. Oxford, England; Pergamon Press, 1993: 363–93.
8. Szeredi L, Schiller I, Sydler T, et al. Intestinal *Chlamydia* in finishing pigs. Vet Pathol 1996; **33**: 369–74.
9. Storz, J. Overview of animal diseases induced by chlamydial infections. In: Barron AL, ed. Microbiology of *Chlamydia*. Boca Raton, Fla: CRC Press Inc., 1988: 167–92.

10. Grimes JE, Arizmendi F, Carter CN, Sneed L. Diagnostic serologic testing of cage and aviary birds for chlamydiosis and suggested confirmatory testing. J Vet Diagn Invest 1996; **8**: 38–44.
11. Buxton D. Potential danger to pregnant women of *Chlamydia psittaci* from sheep. Vet Rec 1986; **118**: 510–1.
12. Jorgensen DM. Gestational psittacosis in a Montana sheep rancher. Emerg Infect Dis 1997; **3**: 191–4.
13. Anderson IE, Baxter SIF, Dunbar S, et al. Analyses of the genomes of chlamydial isolates from ruminants and pigs support the adoption of the new species *Chlamydia pecorum*. Int J Syst Bacteriol 1996; **46**: 245–51.
14. Fukushi H, Hirai K. Proposal of *Chlamydia pecorum* sp. nov. for *Chlamydia* strains derived from ruminants. Int J Syst Bacteriol 1992; **42**: 306–8.
15. Fukushi H, Hirai K. *Chlamydia pecorum*-the fourth species of the genus *Chlamydia*. Microbiol Immunol 1993; **37**: 515–22.
16. Daniels K, Woollen NE, Wilson DJ, Phillips RM, Yeary T. Investigating the link between chlamydia and perinatal morbidity and mortality in swine herds. Vet Med 1994; **2**: 157–62.
17. Nietfeld JC, Janke BH, Leslie-Steen P, Robison DJ, Zeman DH. Small intestinal *Chlamydia* in piglets. J Vet Diagn Invest 1993; **5**: 114–7.
18. Nietfeld JC, Leslie-Steen P, Zeman DH, Nelson D. Prevalence of intestinal chlamydial infection in pigs in the midwest, as determined by immunoperoxidase staining. Am J Vet Res 1997; **58**: 260–4.
19. Rogers DG, Andersen AA, Hogg A, Nilesen DL, Huebert MA. Conjunctivitis and keratoconjunctivitis associated with chlamydiae in swine. JAVMA 1993; **203**: 1321–3.
20. Sarma DK, Tamuli MK, Rahman T, Boro BR, Deka BC, Rajkonwar CK. Isolation of chlamydia from a pig with lesions in the urethra and prostate gland. Vet Rec 1983; **112**: 525.
21. Woolen N, Daniels EK, Yeary T, Leipold HW, Phillips RM. Chlamydial infection and perinatal mortality in a swine herd. JAVMA 1990; **197**: 600–1.
22. Harris JW, Hunter AR, McMartin DA. Experimental chlamydial pneumonia in pigs. Comp Immun Microbiol Infect Dis 1984; **7**: 19–26.
23. Kielstein P, Stellmacher H, Horsch F, Martin J. Zur Chlamydieninfektion des Schweines. 1. Mitteilung: Zur experimentellen Chlamydien-Pneumonie des Schweines. Arch Exper Vet Med 1983; **37**: 569–86.
24. Martin J, Kielstein P, Stellmacher H, Horsch F. Zur Chlamydieninfektion des Schweines. 2. Mitteilung: Pathologisch-histologische Besonderheiten der experimentellen Chlamydienpneumonie des Schweines. Arch Exper Vet Med 1983; **37**: 939–49.
25. Rogers DG, Andersen AA, Hunsaker BD. Lung and nasal lesions caused by a swine chlamydial isolate in gnotobiotic pigs. J Vet Diagn Invest 1993; **8**: 45–55.
26. Vazquez-Cisneros C, Wilsmore AJ, Bollo E. Experimental infections of pregnant sows with ovine *Chlamydia psittaci* strains. Vet Microbiol 1994; **42**: 383–7.
27. Schiller I, Koesters R, Weilenmann R, et al. Mixed infections with porcine *Chlamydia trachomatis/pecorum* and infections with ruminant *Chlamydia psittaci* serovar 1 associated with abortions in swine. Vet Microbiol 1997; **58**: 251–60.
28. Thoma R, Guscetti F, Schiller I, Schmeer N, Corboz L, Pospischil A. Chlamydiae in porcine abortion. Vet Pathol 1997; **34**: 467–9.
29. Zahn I, Szeredi L, Schiller I, et al. Immunhistologischer Nachweis von *Chlamydia psittaci/pecorum* und *C. trachomatis* im Ferkel-Darm. J Vet Med B 1995; **42**: 266–76.
30. Kaltenboeck B, Kousoulas KG, Storz J. Two-step polymerase chain reactions and restriction endonuclease analyses detect and differentiate *ompA* DNA of *Chlamydia* spp. J Clin Microbiol 1992; **30**: 1098–104.
31. Martin JL, Cross GF. Comparison of the *omp1* gene of *Chlamydia psittaci* between isolates in Victorian koalas and other animal species. Aust Vet J 1997; **75**: 579–82.
32. Gimenez D. Staining rickettsiae in yolk sac cultures. Stain Technol 1964; **39**: 135–40.
33. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual, 2nd ed, New York: Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1989.
34. Denamur E, Sayada C, Souriau A, Orfila J, Rodoloakis A, Elion J. Restriction pattern of the major outer-membrane protein gene provides evidence for a homogeneous invasive group among ruminant isolates of *Chlamydia psittaci*. J Gen Microbiol 1991; **137**: 2525–30.
35. Watson MW, Lambden PR, Clarke IN. Genetic diversity and identification of human infection by amplification of the chlamydial 60-kilodalton cysteine-rich outer membrane protein gene. J Clin Microbiol 1991; **29**: 1188–93.
36. Sanger F, Micklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 1977; **74**: 5463–7.
37. Allgemeine Verwaltungsvorschrift über die Durchführung der amtlichen Untersuchungen nach dem Fleischhygienegesetz (VwVFIHG) vom 11.12.1986. (editorial). FlHg 1986.
38. Thiele D, Wittenbrink MM, Fischer D, Krauss H. Evaluation of the polymerase chain reaction (PCR) for detection of *Chlamydia psittaci* in abortion material from ewes. Zbl Bakt 1992; **277**: 446–53.
39. Wittenbrink MM. Nachweis von Antikörpern gegen Chlamydien beim Schwein mit Hilfe eines Immunofluoreszenz- und eines Enzymimmunotests. Berl Münch Tierärztl Wschr 1991; **104**: 270–5.
40. Leonhard I, Wittenbrink MM, Bisping W. Nachweis von *Chlamydia psittaci* im Kot von Schweinen. Berl Münch Tierärztl Wschr 1988; **101**: 124–8.
41. Wittenbrink MM, Wen X, Böhmer N, Amtsberg G, Binder A. Bakteriologische Untersuchungen zum Vorkommen von *Chlamydia psittaci* in Organen von Schweinen und in abortierten Schweinefeten. J Vet Med B 1991; **38**: 411–20.
42. Stellmacher H, Kielstein P, Horsch F, Martin J. Zur

Bedeutung der Chlamydien-Infektion des Schweines unter besonderer Berücksichtigung der Pneumonien. Mh Vet Med 1983; **38**: 601–6.
43. Wendt M, Eggemann G, Wittenbrink M, Jäger C, Weiβ R. Failing K. Prevalence of chlamydial infection in breeding sows. In: Proceedings of the 15th IPVS Congress, Birmingham, England, 5–9 July 1998.
44. Heinen E. Untersuchungen zur Ätiologie von Fruchtbarkeitsstörungen und Atemwegserkrankungen beim Schwein unter besonderer Berücksichtigung von Paramyxoviren und Chlamydien [dissertation]. Giessen, Germany: University of Giessen 1994.
45. Guscetti F, Schiller I, Sydler T, Corboz L, Pospischil A. Experimental *Chlamydia psittaci* serotype 1 enteric infection in gnotobiotic piglets: Histopathological, immunhistochemical and microbiological findings. Vet Microbiol 1998; **62**: 251–63.
46. Griffiths PC, Plater JM, Martin TC, et al. Epizootic bovine abortion in a dairy herd: characterization of a *Chlamydia psittaci* isolate and antibody response. Br Vet J 1995; **151**: 683–93.
47. Schopf K, Khaschabi D, Dackau T. Enzootic abortion in a goat herd, caused by mixed infection with *Coxiella burnetii* and *Chlamydia psittaci*. Case report. Tierärztl Praxis 1991; **19**: 630–4.
48. Wittenbrink MM, Schoon HA, Schoon D, Mansfeld R, Bisping W. Endometritis in cattle experimentally induced by *Chlamydia psittaci*. J Vet Med B 1993; **40**: 437–50.
49. Wittenbrink MM, Kirpal G, Thiele D, Fisher D, Krauss H, Bisping W. Detection of *Chlamydia psittaci* in vaginal discharge of cows: a necessary enlargement of bacteriological diagnosis for the etiologic clarification of fertility disorders in the female cow. J Vet Med B 1994; **41**: 492–503.
50. Quinn TC. DNA amplification assays: a new standard for diagnosis of *Chlamydia trachomatis* infections. Ann Acad Med Singapore 1995; **24**: 627–33.
51. Sayada C, Andersen AA, Storey C, et al. Usefulness of *omp1* restriction mapping for avian *Chlamydia psittaci* isolate differentiation. Res Microbiol 1995; **146**: 155–65.
52. Yang CL, Maclean I, Brunham RC. DNA sequence polymorphism of the *Chlamydia trachomatis omp1* gene. J Infect Dis 1993; **168**: 1225–30.
53. Yoshida H, Kishi Y, Shiga S, Hagiwara T. Differentiation of *Chlamydia* species by combined use of polymerase chain reaction and restriction endonuclease analysis. Microbiol Immun 1998; **42**: 411–4.