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Evidence for Numerous *omp*1 Alleles of Porcine *Chlamydia trachomatis* and Novel Chlamydial Species Obtained by PCR

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A nested PCR for genus-specific amplification of the *Chlamydia omp*1 locus was established. This PCR detected single template molecules in 200-μl specimen aliquots. Amplified chlamydial *omp*1 alleles were typed by heminested species PCRs and allele PCRs. We applied this method to 407 specimens from several host animals with various clinical conditions, and we detected prevalences of chlamydiae from 6 to 50%. Amplicons from peacock enteritis and equine infertility specimens were not typeable according to present *omp*1 allelic criteria for the chlamydial species. DNA sequencing revealed novel *omp*1 alleles which were 29.9 and 47.6% divergent in the deduced peptide sequences from the most closely related chlamydiae. Phylogenetic reconstruction indicated segregation of these alleles from the current four chlamydial species (90 and 97% bootstrap support), thus strongly suggesting the existence of additional chlamydial species. Allele typing of amplicons from swine with intestinal, urogenital, and respiratory infections demonstrated several unique *omp*1 allelic variants of *Chlamydia trachomatis*. These novel alleles had deduced peptide sequences which were 11.6 to 19% divergent from porcine *C. trachomatis* S45. Mutations were clustered in the C-terminal region of variable segment IV of the *omp*1 locus encoding subspecies and serovar determinants of the chlamydial major outer membrane protein, thus implying that there are numerous serovars of porcine *C. trachomatis*. These results demonstrate the need for routine application of sensitive genus-specific detection of chlamydiae in animal specimens and suggest a more prominent role than anticipated for chlamydiae in animal diseases.

Chlamydiae are unique bacteria which exclusively multiply in vacuoles inside eucaryotic host cells (15). Currently, four species, *Chlamydia trachomatis*, *Chlamydia pneumoniae*, *Chlamydia psittaci*, and *Chlamydia pecorum*, are recognized, which generally exhibit low host and disease specificity. In animals, they cause urogenital infections and abortion, enteritis and inapparent intestinal infection, respiratory infections, conjunctivitis, mastitis, polyarthritis, and encephalomyelitis (23). Sensitive diagnosis of chlamydial infections in animals has traditionally relied on cumbersome culture isolation of these organisms. Consequently, it has not been included in routine diagnostic procedures, and chlamydial infections in animals have been, and still are, underdiagnosed (23). Immunological detection of the chlamydial lipopolysaccharide genus antigen has improved routine diagnosis, but sensitivity and specificity of this assay are not fully satisfactory (25).

PCR techniques are considered to be the most sensitive methods of chlamydial detection, and approaches targeting the multicopy chlamydial plasmid are superior to those amplifying chromosomal DNA (14). Since all four chlamydial species can infect animals, *Chlamydia* genus-specific assays are necessary for reliable diagnosis in animals (23). This excludes the plasmid as a target, since it is not present in all chlamydiae (13). We optimized a nested genus-specific PCR for amplification of the chromosomal locus *omp*1 for sensitivity and developed a simple PCR for species or allele typing of amplified chlamydial *omp*1 DNA fragments. In this report, we present data to demonstrate that this PCR, when applied to animal specimens, detected an unexpectedly high number of chlamydial infections caused by known and numerous novel variants of these bacteria.

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MATERIALS AND METHODS

Specimens. DNA isolated from yolk sacs infected with chlamydial prototype strains and from purified *C. psittaci* B577 elementary bodies was used to establish the PCRs (7, 8). Heat-inactivated elementary bodies of the porcine *C. pecorum* isolate Big (*omp*1 genotype LW613) were diluted in fresh milk and used as positive controls and to optimize sensitivity of the nested PCR. Diagnostic animal specimens are listed in Table 1.

DNA extraction. Specimens (approximately 1 mg of tissue or sediment of 0.5 ml of liquid specimens pelleted at 13,000 × *g* for 30 min) were digested with agitation overnight at 55°C in a total volume of 550 μl of lysis buffer (20 mM Tris-HCl [pH 8.0], 1 mM ethylene diamine tetraacetate, 30 mM dithiothreitol, 0.5% sodium dodecyl sulfate, 0.4 mg of proteinase K/ml). DNA was isolated from this digest by cetyltrimethylammonium bromide extraction as previously described (26) and dissolved in 25 μl of distilled water.

Genus-specific nested *Chlamydia omp*1 PCR. The overall strategy of the nested *Chlamydia omp*1 PCR for detection, and of the heminested species- and allele-specific typing PCRs, is depicted in Fig. 1. Fifty-microliter reaction volumes were overlaid with mineral oil, and 50 cycles of 1 s at 97°C, 1 min at 52°C (primary PCR), or 50°C (secondary PCR), and 1 min at 72°C were performed in an ice water-cooled Techne PHC-3 thermal cycler with an internal temperature probe. The reaction buffer in both reactions was 20 mM Tris-HCl (pH 8.3), 25 mM KCl, 2 mM $MgCl_2$, 0.05% Nonidet P-40, 0.05% Tween 20, and 0.01% bovine serum albumin, supplemented with 200 μM deoxynucleoside triphosphate, 0.2 μM each primer (Table 2 and 3), and 1.5 U of *Taq* DNA polymerase (Promega, Madison, Wis.). In all primary reactions, dTTP was substituted with dUTP, 0.5 U of uracil-*N*-glycosylase (Perkin-Elmer Cetus, Norwalk, Conn.) was added, and the reaction mixtures were incubated for 10 min at room temperature prior to cycling (12). This approach resulted in cleavage of contaminating primary amplification products from previous reactions by uracil-*N*-glycosylase and thus prevented carryover contamination. Ten microliters of the extracted specimen DNA was used in primary PCRs, and 2.5 μl of the cycled primary PCRs was used as the template in secondary PCRs. Ten microliters of the secondary reactions was analyzed by 1.5% agarose gel electrophoresis and ethidium bromide staining (21).

In addition to uracil-*N*-glycosylase-mediated control of carryover contamination of primary PCRs, false-positive results were prevented by the following

TABLE 1. Clinical specimens analyzed by *Chlamydia omp*1 PCR

Host (*n*)	Disease(s)	Clinical specimen(s)	No. positive	*omp*1 alleles detected
Peacock (2)	Acute enteritis	Feces	1	Novel species allele
Bird (5)	Acute enteritis, septicemia	Formalin-fixed liver[a]	4	*C. psittaci* MN
Ewe (49)	Herd abortion episode	Placenta, milk, mammary gland biopsy, liver, nasal swab sample, feces[b]	107	*C. psittaci* B577
Calf (3)	Enteritis, polyarthritis	Feces	1	*C. pecorum* LW613
Cow (4)	Chronic mastitis	Milk, mammary gland biopsy	1	*C. psittaci* B577
Mare (6)	Chronic vaginitis, sterility	Vaginal swab sample	1	Novel species allele
Swine (7)	Healthy	Formalin-fixed intestine[a]	2	2 Novel *C. trachomatis*
Swine (2)	Acute postpartal mastitis-metritis	Milk, mammary gland biopsy	1	Novel *C. trachomatis*
Swine (96)	Chronic vaginitis, sterility	Vaginal swab sample	6	Novel *C. trachomatis*
Swine (4)	Acute pneumonia	Lung tissue	2	Novel *C. trachomatis*

[a] *Chlamydia* infection was detected by immunohistochemistry.
[b] Total, 272 specimens.

precautions: (i) use of aerosol-resistant pipette tips in all pre-PCR steps; (ii) strict separation of pre- and post-PCR work areas, dedicated equipment in these laboratories, and frequent changes of gloves; and (iii) overnight UV illumination of pre-PCR areas.

Along with each batch of 12 animal specimens, milk samples with and without 10 inclusion-forming units of Big/ml were processed as positive and negative control reactions, respectively, and 10 genome equivalents of purified B577 DNA dissolved in distilled water was used as a control reaction for PCR performance.

Species- and allele-specific *Chlamydia omp*1 PCRs. For *omp*1 typing of positive amplifications, 5 μl of the 1:100 diluted secondary reactions was used as a template. These heminested typing PCRs (Table 2) were performed in 25-μl volumes with oil overlay for 15 cycles of 1 s at 97°C, 1 min at 50°C, and 1 min at 72°C. Other reaction conditions were identical to those of the secondary PCRs.

DNA sequencing of amplification products. DNA sequence data for amplification products of secondary PCRs were obtained if the products could not be unambiguously typed by species- and allele-specific PCRs. The secondary genus-specific amplification products were purified by 1.5% agarose gel electrophoresis and extraction with Prep-A-Gene (BioRad, Hercules, Calif.). Aliquots of the purified amplicons were subjected to PCR cycle-sequencing reactions with fluorescent dye-labeled dideoxynucleoside triphosphate (Perkin Elmer Cetus) and the secondary genus-specific PCR primers. DNA sequences were determined on an ABI 373A automated DNA sequencer (Perkin Elmer Cetus).

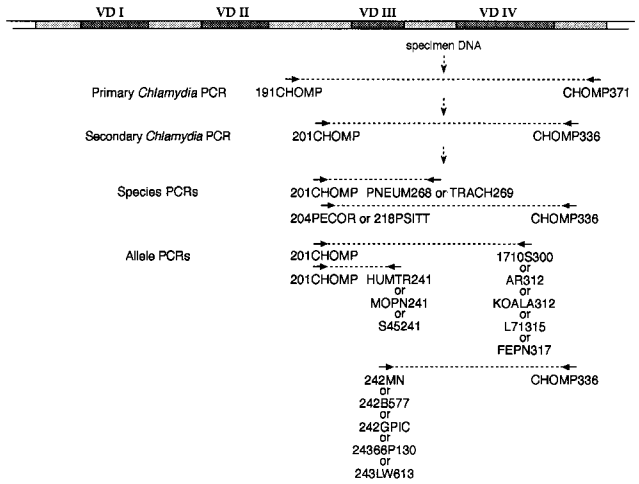

FIG. 1. Schematic diagram of detection and typing of chlamydial DNA by the *Chlamydia omp*1 PCR. The top bar represents the chlamydial *omp*1 gene. The open reading frame is shaded; hypervariable segments within the *omp*1 open reading frame, encoding the MOMP variable domains (VD), are indicated by dark shading. Primers and amplification products are drawn under the *omp*1 bar relative to their position within the *omp*1 open reading frame. Genus-specific primers used in the primary and secondary *Chlamydia* PCRs are targeted towards highly conserved regions between VD II and VD III and after VD IV, respectively. The 3′ ends of the species-specific primers used in the species PCRs are complementary to DNA signature regions for each species, which are located in the constant regions between VD II and VD III (*C. pecorum* and *C. psittaci*) or between VD III and VD IV (*C. pneumoniae* and *C. trachomatis*). The 3′ ends of all allele-specific primers used in the allele PCRs are targeted towards DNA signature regions for the alleles within VD III or VD IV.

Phylogenetic reconstruction. The sequences of novel *Chlamydia omp*1 alleles (Table 1), together with prototypes of all four chlamydial species (Fig. 1), were aligned as peptides with the CLUSTAL W multiple alignment program (24). Optimal results were obtained by alignments with various orders of the taxa and by manual correction. Base sequences were used for phylogenetic analysis of the DNA sequences, and gaps were treated as missing information. Jukes-Cantor distances (6) between pairs of sequences, as determined with the phylogenetic program package PHYLIP (3), identified the optimum evolutionary distance measure for phylogenetic analysis as described by Jin and Nei (5). One thousand bootstrap resamplings of the aligned sequences were performed, and Jin and Nei's distance matrices, with $a = 1$ for the gamma distribution of the substitution rates, were computed (5). This sequence distance measure corrects for substitution rate heterogeneity among sites and is very effective at identifying the correct phylogenetic tree (5). The neighbor-joining algorithm (20) as implemented in PHYLIP was used to identify phylogenetic trees, from which a consensus tree was constructed.

To corroborate the *Chlamydia omp*1 distance tree, we used the branch and bound search option in the program package PAUP (19) to identify a maximum-parsimony phylogenetic hypothesis from the *omp*1 alignment.

Nucleotide sequence accession numbers. Sequence accession numbers in GenBank are as follows: mare vaginitis chlamydial DNA, U82954; peacock enteritis chlamydial DNA, U82955; swine intestinal infection chlamydial DNA 1, U82956; swine intestinal infection chlamydial DNA 2, U82957; swine mastitis chlamydial DNA, U82958; swine vaginitis chlamydial DNA, U82959; and swine pneumonia chlamydial DNA, U82960.

RESULTS

Chlamydial DNA detected and typed by *Chlamydia omp*1 PCRs. In preliminary experiments to maximize the sensitivity of a nested *Chlamydia omp*1 PCR (7) for clinical specimens, we found that substances coextracted with DNA from clinical specimens inhibited the *omp*1 PCR in a target-length-dependent manner (18). Reducing the size of the amplicon in a modified nested *Chlamydia omp*1 PCR restored the sensitivity. This PCR visibly amplified single target molecules from whole chlamydial organisms when these were added to test specimens which yielded a background of as much as 1 μg of unrelated DNA (data not shown). We used 50 cycles of primary and secondary genus-specific PCR to overcome some residual inhibition found in many clinical specimens.

To confirm the genus-specific amplification of the chlamydial *omp*1 locus, we amplified DNA of representative strains from all four chlamydial species (7, 8). All chlamydial strains, but not *Chlamydia*-negative DNA, yielded intensely staining DNA fragments of the expected sizes in the secondary *Chlamydia omp*1 PCR (Fig. 2, upper panel).

An approach for differentiation of amplified secondary DNA fragments was designed. This PCR typing approach is based on efficient extension from perfectly matched primers

TABLE 2. Primers used in PCRs for detection and typing of the chlamydial *omp*1 locus and amplification products of the reactions

PCR (primers)	*C. trachomatis*[a]			*C. pneumoniae*		*C. psittaci*				*C. pecorum*			
	LGV2	MOPN	S45	AR388	KOALA	MN	B577	GPIC	FEPN	66P130	1710S	L71	LW613
Genus specific													
Primary *Chlamydia* (191CHOMP + CHOMP371)	585	576	588	582	582	597	582	588	588	594	582	579	588
Secondary *Chlamydia* (201CHOMP + CHOMP336)	443	434	446	440	440	455	440	446	446	452	440	437	446
Species specific													
C. trachomatis (201CHOMP + TRACH269)	250	250	250										
C. pneumoniae (201CHOMP + PNEUM268)				244	244								
C. psittaci (218PSITT + CHOMP336)						404	389	395	395				
C. pecorum (204PECOR + CHOMP336)										441	429	426	435
Allele specific													
Human *C. trachomatis*[b] (201 CHOMP + HUMTR241)	162												
C. trachomatis MOPN (201CHOMP + MOPN241)		163											
C. trachomatis S45 (201CHOMP + S45241)			162										
C. pneumoniae AR388 (201CHOMP + AR388312)				375									
C. pneumoniae KOALA (201CHOMP + KOALA312)					376								
C. psittaci MN (242MN + CHOMP336)						329							
C. psittaci B577 (242B577 + CHOMP336)							320						
C. psittaci GPIC (242GPIC + CHOMP336)								319					
C. psittaci FEPN (201CHOMP + FEPN317)									398				
C. pecorum 66P130 (24366P130 + CHOMP336)										323			
C. pecorum 1710S (201CHOMP + 1710S300)											340		
C. pecorum L71 (201CHOMP + L71315)												384	
C. pecorum LW613 (243LW613 + CHOMP336)													318

[a] Numbers are the size (in base pairs) of the amplification product for each reaction.
[b] Amplifies all human *C. trachomatis omp*1 alleles.

versus inefficient extension from 3′-mismatched primers (2, 11, 17). Positive secondary genus-specific reactions were diluted 1:100, and 5 μl of each of these dilutions containing approximately 10^9 molecules of the *omp*1 amplicon was amplified in 15 PCR cycles. The reactions contained one of the secondary genus-specific primers and one primer specific for the DNA signature sequence of a chlamydial species (species PCR) or for a single chlamydial *omp*1 allele (allele PCR) (Table 2 and

TABLE 3. Oligonucleotide primers used for amplification of the *Chlamydia omp*1 locus

PCR	Primer[a]	Description[b]
Genus specific		
Chlamydia spp.	191CHOMP	5′-GCI YTI TGG GAR TGY GGI TGY GCI AC-3′
Chlamydia spp.	201CHOMP	5′-GGI GCW GMI TTC CAA TAY GCI CAR TC-3′
Chlamydia spp.	CHOMP336	5′-CAA GMT TTT CTG GAY TTM AWY TTG TT-3′
Chlamydia spp.	CHOMP371	5′-TTA GAA ICK GAA TTG IGC RTT IAY GTG IGC IGC-3′
Species specific		
C. trachomatis	TRACH269	5′-ACC ATT TAA CTC CAA TGT ARG GAG TG-3′
C. pneumoniae	PNEUM268	5′-GTA CTC CAA TGT ATG GCA CTA AAG A-3′
C. psittaci	218PSITT	5′-GTA ATT TCI AGC CCA GCA CAA TTY GTG-3′
C. pecorum	204PECOR	5′-CCA ATA YGC ACA ATC KAA ACC TCG C-3′
Allele specific		
C. trachomatis human serovars	HUMTR241	5′-ATC YTT AGT YCC YGT CGC AGC WTC-3′
C. trachomatis MOPN	MOPN241	5′-CAT CTT TAG TAT CTG TAG CGC TAA C-3′
C. trachomatis S45	S45241	5′-ATC TTT AGT CCC TGT AGC AGC AGT-3′
C. pneumoniae AR388	AR388312	5′-ATC AGT AGT AGA CAA TGC TGT GGC-3′
C. pneumoniae KOALA	KOALA312	5′-AAT CAC TAG TAG GCA ATG TTG TCG T-3′
C. psittaci MN	242MN	5′-CCT ATA ACG GCT GGA ACA ACA GAA-3′
C. psittaci B577	242B577	5′-CCT CTA ACA GCT GGT ACT GAT CAG-3′
C. psittaci GPIC	242GPIC	5′-CCT TAA CCG CTG GAA CAG AGA GC-3′
C. psittaci FEPN	FEPN317	5′-ATG AAG TCA GCA TAT TTG TTG GAG G-3′
C. pecorum 66P130	24366P130	5′-GCT GGA ACA AGC ACA GAC ACT GC-3′
C. pecorum 1710S	1710S300	5′-ATG TAG GAT TCC ATG TAG TCA GCC-3′
C. pecorum L71	L71315	5′-GTC AGC ATA TTT GTC GCT TCC TAC-3′
C. pecorum LW613	243LW613	5′-TGC AGG AAC GGA AAC TGA TTC TAG-3′

[a] The first or last three digits in the primer designation indicate the codon position of the 3′ end of the primer relative to the MN *omp*1 open reading frame (27). The position of the three-digit number to the left of the letters indicates priming in the sense direction, and a position to the right indicates priming in the antisense direction. The letters indicate specificity for the *omp*1 locus of the genus *Chlamydia* (CHOMP) or for the designated chlamydial species or strain represented by the *omp*1 allele. The approximate positions of the primers relative to the *omp*1 variable domains are indicated in Fig. 1. Primers 191CHOMP, CHOMP336, and CHOMP371 were described previously (7, 8); all other primers were designed according to sequences and alignments published by Kaltenboeck et al. (8) and Zhang et al. (27).

[b] Degenerate positions are indicated as follows: I, inosine; K, G or T; M, A or C; R, A or G; W, A or T; and Y, C or T.

3). These heminested PCRs efficiently amplified cognate *omp*1 alleles (Fig. 2, lower four panels) but not 3′-mismatched alleles, which required 20 or more PCR cycles for visible amplification due to inefficient primer extension (data not shown).

Numerous nontypeable chlamydial *omp*1 alleles found by the *omp*1 PCRs. We evaluated the nested *Chlamydia omp*1 PCR on 407 specimens from different hosts and disease manifestations (Table 1). Chlamydial DNA was detected in all types of specimens with surprisingly high frequency, while the negative control specimens always remained *Chlamydia* negative. Representative positive secondary amplifications are shown in Fig. 3. Only specimens from ewe abortion, ewe rhinitis, and ewe mastitis, obtained from an episode of ovine herd abortion, yielded visible specific DNA fragments in the primary amplification, indicating a high number of targets (data not shown). In the secondary PCRs, all specimens exhibited intensely stained, specific DNA fragments of slightly different sizes (Fig. 3, upper panel).

We determined the *omp*1 alleles of these amplicons by species PCRs (Fig. 3, lower four panels) and allele PCRs (Fig. 4). *C. trachomatis* species PCRs, but not the other species PCRs, of all porcine specimens (infected with alleles swine intestinal 1 and 2, swine mastitis, swine vaginitis, and swine pneumonia) were positive (Fig. 3). Specimens from cases of bird septicemia, cow mastitis, ewe abortion, ewe rhinitis, and ewe mastitis were positive only in the *C. psittaci* species PCRs, while the calf intestinal specimen was positive only in the *C. pecorum* species PCR. The *omp*1 amplicons of the positive specimens infected with peacock enteritis and mare vaginitis did not yield any positive species PCRs, suggesting that they might be from as-yet-unrecognized chlamydial species.

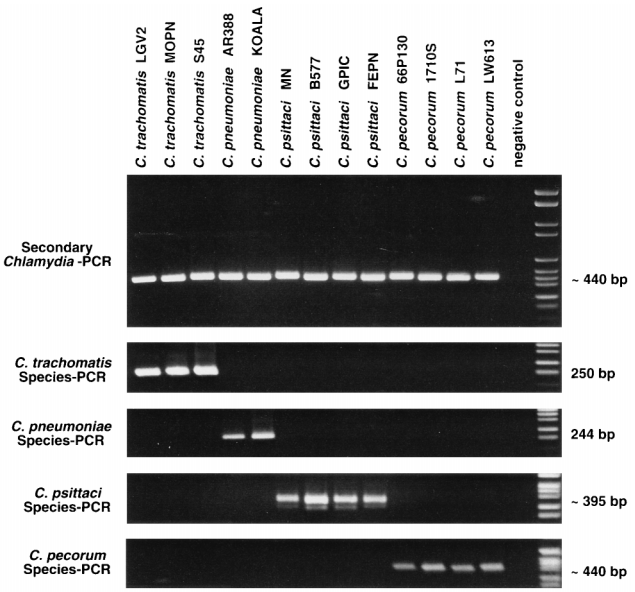

FIG. 2. Amplification of the *omp*1 locus of all chlamydial species by the *Chlamydia omp*1 PCR. DNA of yolk sac tissue infected with prototypes of the four chlamydial species was amplified with nested *Chlamydia omp*1 PCRs, and the secondary reactions were analyzed by 1.5% agarose gel electrophoresis and ethidium bromide staining (upper panel). Aliquots of secondary PCRs were subjected to 15 cycles of species PCRs specific for chlamydial species (four lower panels). The molecular size marker (right lane) is ϕX174 DNA restricted with *Hae*III.

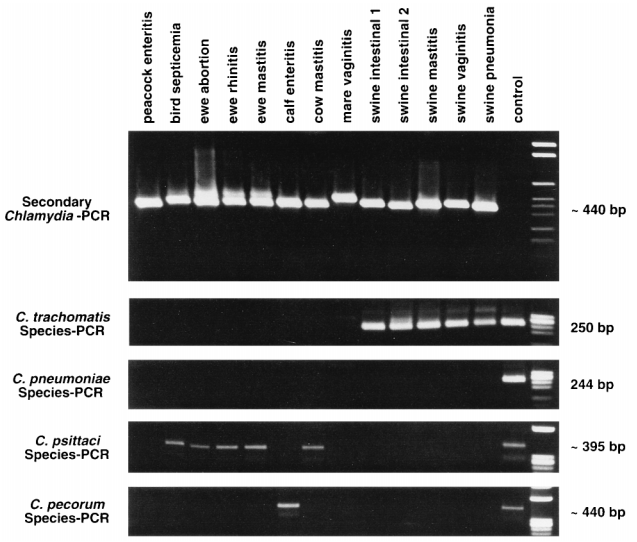

FIG. 3. Representative positive results of the *Chlamydia omp*1 PCR of clinical specimens. Secondary reactions of the genus-specific *Chlamydia omp*1 PCRs of DNA extracted from specimens are shown in the upper panel, and species PCRs are shown in the lower four panels. Molecular size markers are shown in the right lane.

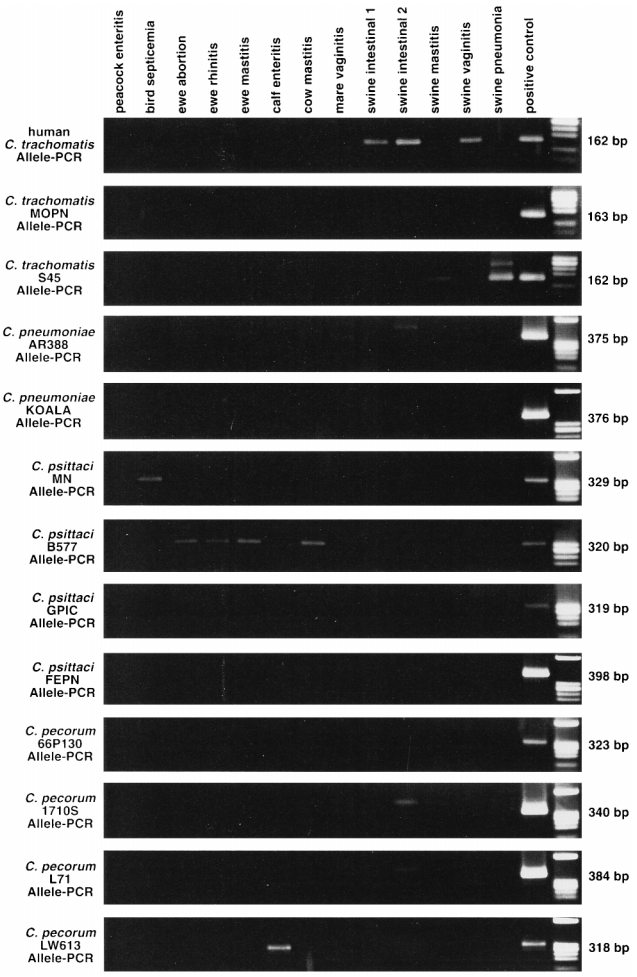

FIG. 4. Allele PCRs of *Chlamydia*-positive clinical specimens. Aliquots of positive secondary genus-specific PCRs from clinical specimens were subjected to 15 cycles of allele PCRs specific for each of the indicated *omp*1 types and were separated by 1.5% agarose gel electrophoresis. Molecular size markers are shown in the far right lane.

Allele PCRs of these positive secondary reactions yielded results consistent with the species PCRs (Fig. 3). Weak, aberrant DNA fragments were observed in some allele PCRs containing the genus-specific primer 201CHOMP (e.g., PCRs of *C. pneumoniae* AR388, *C. pneumoniae* KOALA, *C. psittaci* FEPN, *C. pecorum* 1710S, and *C. pecorum* L71), but comparison with the positive control unequivocally identified specific amplification in these allele PCRs. The specimen from bird septicemia was positive only in the *C. psittaci* MN allele PCR, the specimen from calf enteritis was positive only in the *C. pecorum* LW613 reaction, and the specimens from cases of ewe abortion, ewe rhinitis, ewe mastitis, and cow mastitis were positive only in the *C. psittaci* B577 allele PCR. The chlamydial *omp*1 alleles amplified in these PCRs could therefore be unequivocally identified. Secondary genus-specific products of peacock enteritis and mare vaginitis did not yield a specific positive amplification in any of the allele PCRs and thus could not be typed with the present approach based on known chlamydial *omp*1 DNA sequences.

Porcine specimens from cases of swine mastitis, swine vaginitis, and swine pneumonia were positive in various intensities in both the allele PCR for human serovars of *C. trachomatis* and that for porcine *C. trachomatis* S45, while the specimens from cases of swine intestinal infections 1 and 2 yielded specific products which stained less intensely than the positive control amplification only in the human *C. trachomatis* allele PCR. These results prevented unequivocal allele identification and presented evidence for the existence of novel *C. trachomatis omp*1 alleles, indicating that DNA sequencing was necessary for precise allele determination.

DNA sequencing identifies numerous novel porcine *C. trachomatis* bacteria and putative new chlamydial species. Sequencing of the nontypeable amplicons from swine and from specimens infected with peacock enteritis and mare vaginitis revealed that the deduced amino acid sequences differed substantially from all known *Chlamydia omp*1 alleles (Fig. 5). The *omp*1 locus most closely related to all swine amplicons was from porcine *C. trachomatis* S45 (8), from which they differed by 11.6 (swine intestinal infection 1) to 19% (swine vaginitis). The differences from S45 clustered mostly in the C-terminal portion of variable segment IV, which encodes subspecies and serovar determinants of the chlamydial major outer membrane protein (MOMP) (1, 22). The peacock intestinal amplification product was closest to *C. pneumoniae* AR388 (29.9% sequence divergence), and the mare vaginitis amplicon was closest to *C. pecorum* 66P130 (47.6% sequence divergence). The 147-amino-acid-position (including gaps) alignment of the deduced amino acid sequences revealed in the mare vaginitis amplicon a 12-amino-acid insertion near the N-terminus of variable domain IV (Fig. 5) which is not present in any other *Chlamydia omp*1 allele. This explained the large size of the secondary genus-specific amplification product as visualized in Fig. 3.

Phylogenetic reconstruction with the neighbor-joining algorithm (20) and the Jin-Nei (5) distance matrix of 1,000 bootstrap replicates of the 441-nucleotide alignment yielded a phylogenetic consensus tree (Fig. 6), which was largely congruent with a previous phylogenetic analysis of a larger portion of the chlamydial *omp*1 locus (8). In particular, separations between chlamydial species and most of the branches within species were identical, and only minor differences in the branching order within *C. psittaci* and *C. trachomatis* were observed. A

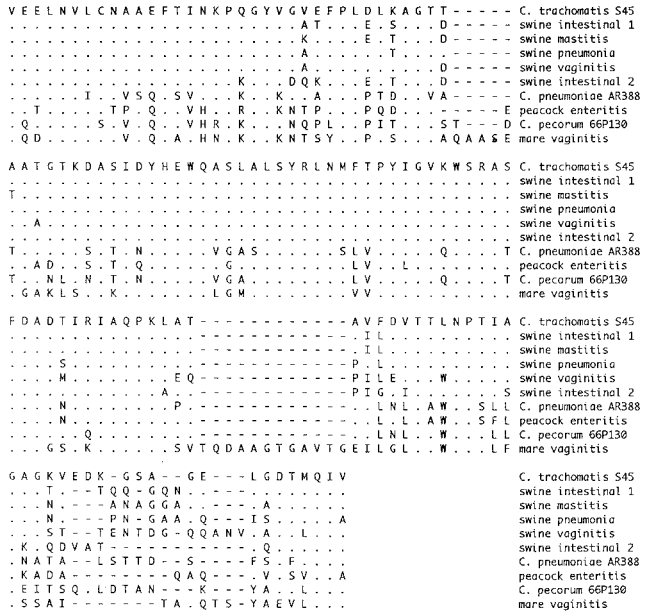

FIG. 5. Alignment of deduced amino acid sequences of the novel *Chlamydia omp*1 alleles. Dots represent amino acids identical to the *C. trachomatis* S45 sequence. Novel alleles are shown under the most similar chlamydial prototypes (*C. trachomatis* S45, *C. pneumoniae* AR388, and *C. pecorum* 66P130). S45-MOMP variable domain III, VEFPLDLKAGTTAATGT (amino acid positions 228 to 246 in the *C. psittaci* MN *omp*1 [27] open reading frame); S45-MOMP variable domain IV, LATAVFDVTTLNPTIAGAGKVEDKGSAGELG (amino acid positions 294 to 325 in the *C. psittaci* MN *omp*1 open reading frame).

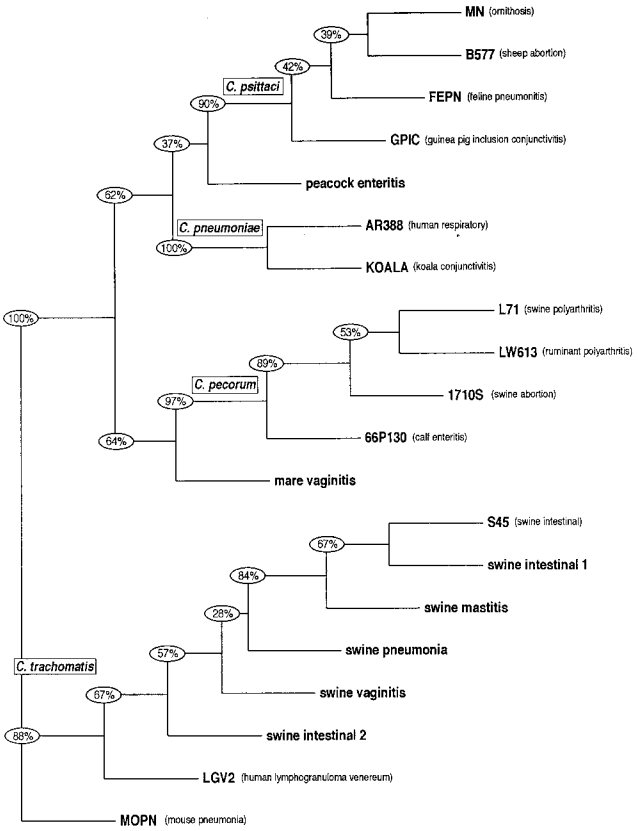

FIG. 6. Unrooted consensus phylogram of part of the chlamydial *omp*1 locus inferred by the neighbor-joining algorithm. Jin-Nei distance matrices computed from 1,000 bootstrap replicates of the aligned DNA sequences were used to construct the phylogenetic tree. For output purposes, *C. trachomatis* MOPN was assumed to be the outgroup. Branch lengths are proportional to the calculated average substitution rate per nucleotide site (distance), except for terminal branches. Numbers in ovals indicate the percent of neighbor-joining bootstrap replicates that support the topological elements to the right of the oval (bootstrap support). Chlamydial species are indicated in boxes above the segregating branch, and disease association is listed in brackets after the allele designations of the prototype strains.

maximum-parsimony tree (1,034 steps; consistency index, 0.494; retention index, 0.583) constructed from the same alignment had a topology similar to the neighbor-joining tree with minor differences in branches within *C. psittaci* and *C. trachomatis*, which had weak bootstrap support of less than 40% in the neighbor-joining consensus tree. Interestingly, the peacock enteritis and mare vaginitis chlamydial alleles did not cosegregate with any of the current chlamydial species. Separation from the closest species was strongly bootstrap supported for both alleles: the peacock enteritis chlamydial allele was at 90 and 100% support distinct from *C. psittaci* and *C. pneumoniae*, and the mare vaginitis chlamydial allele was at 97% from *C. pecorum* and at 100% from both *C. pneumoniae* and *C. trachomatis*. These results conform with the dissimilarity between species-specific primers and the homologous *omp*1 sequences of these alleles and those of the negative species PCRs (Fig. 3).

The novel *omp*1 alleles amplified from swine specimens all clustered within *C. trachomatis*, between porcine allele S45 and human allele LGV2 from serovar lymphogranuloma venereum 2. Again, these data conform with the results of species and allele PCRs (Fig. 3 and 4).

DISCUSSION

We have improved a PCR for efficient amplification of the chlamydial *omp*1 target in specimens containing inhibitors of the PCR. Species- and allele-specific primers then differentiated the amplified DNA fragments. Application of these techniques to animal specimens detected a surprisingly high prevalence of chlamydiae in various host animals and diseases. Moreover, species and allele PCR typing methods unequivocally identified the *omp*1 alleles MN, B577, and LW613 among the amplicons and suggested the existence of novel chlamydial species, represented by the peacock enteritis and mare vaginitis chlamydial alleles, and a high prevalence of as-yet-untypeable variants of *C. trachomatis* in swine.

Contamination of PCRs with previously amplified DNA is a major concern in diagnostic applications like the *Chlamydia omp*1 PCR, which can detect single target molecules (10). We have established a net of precautions against this PCR product carryover. The detection of numerous highly divergent *Chlamydia omp*1 alleles, and the fact that we did not observe any falsely positive amplification in negative control reactions, suggests successful contamination control.

For convenient and reliable differentiation of amplification products, we designed species- and allele-specific primers which hybridize to all amplified *omp*1 alleles but which have one or more mismatches to noncognate amplicons at the 3′ ends. In conjunction with a genus-specific primer, they amplify in the species and allele PCRs specific DNA fragments from cognate alleles of saturated positive secondary genus-specific PCRs. While the efficient extension of perfectly 3′-matched primers results in visible amplification after 15 thermal cycles, products from noncognate alleles begin to appear only after 20 to 25 PCR cycles. We have found this approach very reliable, with the advantage that primers for novel alleles can easily be developed.

The identification of numerous and novel chlamydial *omp*1 alleles in many specimens from a variety of host species and disease conditions validates the efforts to optimize PCR sensitivity and ease of allele differentiation. While the amplification of *omp*1 alleles MN, B577, and LW613, and the associated clinical manifestations, conforms with a large body of research (reviewed in reference 23), the detection of several novel alleles of *C. trachomatis* in swine and of two non-species-typeable alleles was unexpected. The positions of the majority of the sequence divergences of the porcine alleles in serovar-determining regions suggests the existence of numerous serovars of *C. trachomatis* in swine. The notion of the existence of additional chlamydial species, as exemplified by peacock enteritis and mare vaginitis chlamydial alleles, is corroborated by the strong bootstrap support of the segregation of these alleles from the current chlamydial species.

These findings, obtained with genus-specific amplification, suggest that our understanding of animal chlamydial infections is biased towards acute disease caused by readily cultivable chlamydial strains. The recognition, over the last decade, of the importance of *C. pneumoniae* as a ubiquitous human pathogen (4) could be a paradigm for future developments in the knowledge about animal chlamydiae. Wide application of genus-specific PCR might reveal a high prevalence of numerous chlamydial variants in animals and a more subtle association with disease, similar to the involvement of *C. pneumoniae* in human coronary heart disease (9, 16).

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