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Two-Step Polymerase Chain Reactions and Restriction Endonuclease Analyses Detect and Differentiate *ompA* DNA of *Chlamydia* spp.

BERNHARD KALTENBOECK,† KONSTANTIN G. KOUSOULAS, AND JOHANNES STORZ*

Department of Veterinary Microbiology and Parasitology, School of Veterinary Medicine, Louisiana State University, Baton Rouge, Louisiana 70803

Received 22 July 1991/Accepted 3 February 1992

Specific and sensitive amplification of major outer membrane protein (MOMP) gene (*ompA*) DNA sequences of *Chlamydia* species with various MOMP genotypes was achieved by a two-step polymerase chain reaction (PCR). Degenerate, inosine-containing oligonucleotide primers homologous to the 5′ and 3′ ends of the translated regions of all chlamydial MOMP genes were used in a PCR to amplify a DNA fragment of approximately 1,120 bp. A portion of this DNA fragment was amplified in a second genus-specific reaction that yielded a DNA fragment of approximately 930 bp. A pair of degenerate oligonucleotide primers homologous to internal sequences of the primary DNA fragment was used in this PCR. This method detected three cognate chlamydial genomes in a background of 1 μg of unrelated DNA. MOMP genes of 13 representative chlamydial MOMP genotypes of the species *C. trachomatis*, *C. pneumoniae*, and *C. psittaci* were amplified. In a secondary PCR, group-specific detection was achieved by the simultaneous use of one genus-specific primer and three primers derived from different fingerprint regions of three major groups of chlamydiae. This multiplex PCR differentiated the groups by the length of the amplified DNA fragments and detected the simultaneous presence of DNA sequences of the *Chlamydia* spp. with different MOMP genotypes. Further differentiation as *ompA* restriction fragment length polymorphism types among all chlamydial strains with the various MOMP genotypes analyzed here was achieved by restriction endonuclease analysis of the secondary PCR products. DNA sequences corresponding to the *ompA* restriction fragment length polymorphism type B577 of *C. psittaci* were detected in two of seven milk samples from cases of bovine mastitis.

Chlamydiae are widespread intracellular bacteria that cause clinically inapparent infections and a variety of diseases in humans, marsupials, other mammals, and birds. In humans, the most notable diseases are trachoma and urogenital infections caused by *C. trachomatis*, respiratory infections caused by *C. pneumoniae*, and psittacosis caused by *C. psittaci* (9, 22). In animals, *C. psittaci* is capable of inducing diverse disease syndromes like enteritis, urogenital infections and abortions, pneumonia, polyarthritis, polyserositis, encephalitis, and mastitis (25).

These agents are detected by cell culture isolation, enzyme-linked immunosorbent assay methods for chlamydial antigen identification, and in situ hybridization (23). Recently, sensitive polymerase chain reaction (PCR) methods were reported for the detection of DNA sequences of the common plasmid of *C. trachomatis* (5, 19) and the major outer membrane protein (MOMP) genes of *C. trachomatis*, *C. pneumoniae*, and *C. psittaci* (10). While the species *C. trachomatis* and *C. pneumoniae* were examined comprehensively (10), amplification of the highly heterogeneous species *C. psittaci* was analyzed by using only one representative strain. Thus, it is uncertain which variants of *C. psittaci* can be detected by PCR.

Recently, we obtained partial (81%) MOMP gene DNA sequences of 25 representative chlamydial strains using PCR. On the basis of the sequences of the *ompA* loci from different strains, phylogenetic relationships among the chlamydiae were analyzed (12). Four groups of MOMP genotypes were found. *C. trachomatis* with the porcine chlamydial isolate S45 made up the trachoma group. *C. pneumoniae* combined with the MOMP genotype KOALA of isolates from the koala (*Phascolarctos cinereus*) formed the pneumonia group. A cluster of *C. psittaci* strains from ruminant and porcine hosts with a propensity for causing polyarthritis-polyserositis was termed the polyarthritis group. Strains of *C. psittaci* originating from avian, human, ruminant, feline, and guinea pig hosts made up the psittacosis group.

On the basis of these findings, we explored the possibility of sensitive genus-specific detection of *Chlamydia* spp. by PCR. We report here the detection of *Chlamydia* spp. by PCR amplification of MOMP gene DNA sequences, the PCR-based classification of chlamydial MOMP genes into four major groups, the differentiation of *ompA* restriction fragment length polymorphism (RFLP) types within these groups by restriction endonuclease analysis, and the detection of *C. psittaci* in clinical specimens.

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MATERIALS AND METHODS

Chlamydial strains. Thirteen strains of *Chlamydia* representing 13 MOMP genotypes; at least nine serovars; and six genomic RFLP types of human, koala, bovine, ovine, porcine, feline, guinea pig, and murine origin (7, 12, 20) were used in this investigation. Relevant information about the isolates is summarized in Table 1. All chlamydial strains

* Corresponding author.
† Present address: II. Medizinische Universitätsklinik für Klauentiere, Veterinärmedizinische Universität Wien, Linke Bahngasse 11, A-1030 Vienna, Austria.

TABLE 1. Origin, MOMP genotype, serovar, and RFLP type of chlamydial strains included in detection and differentiation of chlamydial MOMP genes by PCR

Chlamydial strain	MOMP genotype[a]	Serovar[b]	RFLP type[c]	Host	Host clinical condition	Reference
C. pneumoniae AR388	AR388			Human	Respiratory infection	3
C. psittaci						
KOALA	KOALA			Koala	Keratoconjunctivitis	8
FEPN	FEPN	7	Feline 1	Cat	Live vaccine	20
GPIC	GPIC	8		Guinea pig	Conjunctivitis	20
B577	B577	1	Avian 2b	Sheep	Abortion	20
BMZ1121	MN		Avian 1b	Cattle	Mastitis	14
C. trachomatis						
MOPN	MOPN		Mouse	Mouse	Pneumonia	18
L2	L2	L2	Human	Human	Lymphogranuloma venereum	26
S45	S45	5		Swine	Inapparent intestinal infection	20
C. psittaci						
66P130	66P130	3		Cattle	Inapparent intestinal infection	20
LW613	LW613	2	Ruminant 1	Cattle	Polyarthritis	20
L71	L71	4		Swine	Polyarthritis	20
1710S	1710S	6		Swine	Abortion	20

[a] MOMP genotypes were determined by Kaltenboeck et al. (12).
[b] Serovars of *C. psittaci* were determined by Perez-Martinez and Storz (20); the serovar of *C. trachomatis* L2 was determined by Wang and Grayston (26).
[c] RFLP types were determined by Fukushi and Hirai (7).

except *C. pneumoniae* AR388 and *C. psittaci* KOALA were propagated in developing chicken embryos as described previously (20). In addition, strain B577 was also cultivated in persistently infected L cells (21).

Extraction of chlamydial DNA. Heavily infected yolk sacs and purified B577 elementary bodies were used for the isolation of chlamydial DNA by cetyltrimethylammonium bromide extraction as described previously (13). Purified DNAs from *C. pneumoniae* AR388 and *C. psittaci* KOALA were kind gifts of L. A. Campbell, University of Washington, Seattle, and Adeeb A. Girjes, University of Queensland, Brisbane, Queensland, Australia, respectively.

Chlamydia-negative background DNAs from uninfected L cells and bacteria likely to be encountered in clinical specimens (*Escherichia coli*, *Salmonella cholerae-suis*, *Bordetella bronchiseptica*, *Pseudomonas aeruginosa*) were extracted after low-speed sedimentation as described previously (13).

Oligonucleotide primers. A schematic representation of the chlamydial MOMP gene (now termed *ompA* [27]), the relative locations of the primers, and the DNA fragments generated in the PCRs are presented in Fig. 1. The primers are described in Table 2. These degenerate, inosine-containing oligonucleotide primers (17) were synthesized in our laboratory by the phosphoramidite method on a Gene Assembler Plus Synthesizer (Pharmacia-LKB, Piscataway, N.J.) and were used without further purification. With the exception of TROMP358, PNOMP268, and PSOMP204, these oligonucleotide primers hybridized to DNA sequences of the MOMP gene of all chlamydial strains examined. TROMP358 is specific for the trachoma group of chlamydial MOMP genotypes, PNOMP268 is specific for the pneumonia group, and PSOMP204 is specific for the polyarthritis group. These primers were designed to match the known target sequences of their cognate chlamydial group, but clustered at the 3′ ends they contain as many as eight nucleotides which do not match the MOMP genes of the other chlamydial groups. When they were combined in one PCR with the

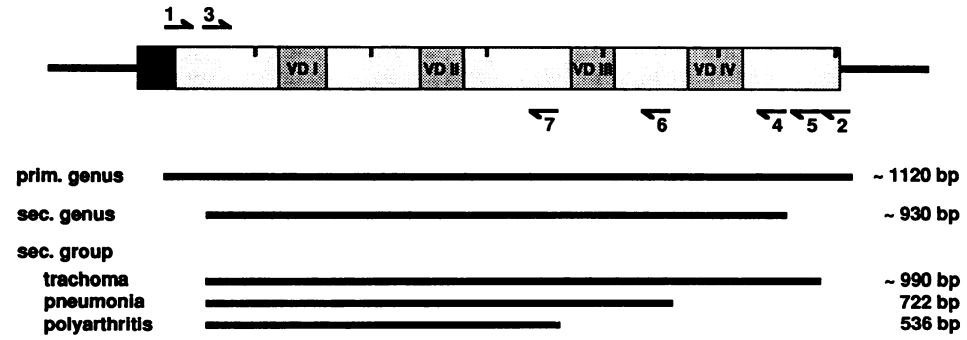

FIG. 1. Schematic representation of the chlamydial MOMP gene indicating the relative positions of the oligonucleotide primers used in the PCR amplifications. The translated regions are boxed; the leader peptide is darkly shadowed. Variable domains encoding antigenic determinants are interspersed among highly conserved regions. Primers are drawn to scale only at the 3′ ends. DNA fragments generated in the PCR amplifications are indicated below the MOMP gene representation. Primers 1 and 2 (9CHOMP and CHOMP371) were used in chlamydia genus-specific primary amplification (prim. genus); primers 3 and 4 (29CHOMP and CHOMP336) were used in genus-specific secondary amplification (sec. genus). Primer 5 (TROMP358) is specific for the trachoma group of chlamydial MOMP genotypes, primer 6 (PNOMP268) is specific for the pneumonia group, and primer 7 (PSOMP204) is specific for the polyarthritis group. These primers were used together with primer 3 (29CHOMP) in the secondary group-specific amplification (sec. group).

TABLE 2. Oligonucleotide primers used in primary and secondary PCR amplifications of chlamydial MOMP genes

Primer[a]	Sequence	Position[b]
9CHOMP	5′-GCI(CT)TGCCTGTIGGGAA(CT)CCIGCIGA(AG)CC-3′	64–92
CHOMP371	5′-TTAGAAIC(GT)GAATTGIGC(AG)TTIA(TC)GTGIGCIGC-3′	+3–1177
29CHOMP	5′-GGIGA(CT)CCITG(CT)GA(CT)CCITG-3′	133–152
CHOMP336	5′-CAAG(AC)TTTTCTGGA(CT)TT(AC)A(AT)(CT)TTGTT-3′	1097–1072
TROMP358	5′-G(AT)GTCTCAA(CT)(AT)GTAACTGC(AG)T-3′	1159–1139
PNOMP268	5′-CCAATGTATGGCACTAAAGA-3′	887–868
PSOMP204	5′-ACGTT(GT)A(AG)TTCTTGAACGCG-3′	695–676

[a] Primer designations: OMP, outer membrane protein gene; CH, *Chlamydia* spp.; TR, trachoma; PN, pneumonia; PS, psittacosis. The number in the primer designation indicates the codon of *C. psittaci* MN (28) at the 3′ end of the primer, and the position of the number to the left or right of the letters identifies sense or antisense priming, respectively.

[b] Position of the primer (5′ to 3′) on the MOMP gene of *C. psittaci* MN. +, 3′-nontranslated regions of the MOMP gene.

genus-specific primer 29CHOMP, they generated DNA fragments of different lengths characteristic of their respective chlamydial *ompA* target (Fig. 1).

PCR amplification. All PCRs were performed with 2.5 U of *Taq* DNA polymerase (Gibco-Bethesda Research Laboratories, Gaithersburg, Md.) in 100-μl reactions containing 0.01% bovine serum albumin and 0.1% Tween 20 in 20 mM Tris-HCl (pH 8.3)–2.0 mM $MgCl_2$–25 mM KCl–200 μM (each) deoxynucleoside triphosphates. Primers 9CHOMP and CHOMP371 were used at 0.2 μM each in *Chlamydia* genus-specific primary amplification; primers 29CHOMP and CHOMP336 were used in genus-specific secondary amplification. Primers TROMP358, PNOMP268, and PSOMP204 were used together with 29CHOMP at 0.1 μM each in the secondary group-specific amplification. To avoid PCR product carryover, positive displacement pipettes were used, and DNA was extracted and PCRs were assembled in two different laboratories, which were strictly kept free of amplification products. The samples were overlaid with mineral oil; and in the primary amplification, they were subjected to 40 cycles of 1 s at 96°C, 30 s at 56°C, and 1 min at 72°C in a programmable DNA thermal cycler (Eppendorf, Fremont, Calif.). Secondary amplifications were performed for 27 cycles of 1 s at 96°C, 30 s at 52°C, and 1 min at 72°C. After primary and secondary PCRs, the samples were incubated at 72°C for 7 min. The sensitivity of the PCR was established by using various amounts of purified B577 DNA in a background of 1.0 μg of a mixture of uninfected L-cell and bacterial DNAs as input for primary amplification. Amounts of 5 μl of amplified primary reactions were used as input DNA in the secondary PCRs. Two negative controls consisting of DNA extracted from normal L cells and milk samples were included in all amplifications. A third control reaction contained only distilled water (no DNA). PCR screening of samples was performed by using 30 cycles for the PCRs and 10 μl of 1:100 diluted DNA (30 to 100 ng) extracted from infected yolk sacs or purified chlamydial DNA. Subsequently, 5 μl of the 10^{-3}-diluted primary PCR products were used in the secondary genus- and group-specific PCRs.

Analysis of amplified DNA. Amounts of 10 μl of the amplified reaction were fractionated by 1.5% (1:1 ratio of DNA-grade agarose to NuSieve agarose [FMC BioProducts, Rockland, Maine]) or 4% (1:3 ratio of DNA-grade agarose to NuSieve agarose) agarose gel electrophoresis directly or after restriction endonuclease digestion, respectively. The DNA was visualized by ethidium bromide staining and UV fluorescence. The chlamydial MOMP genotypes were differentiated by restriction endonuclease analysis of the secondary amplification products. Genus-specific DNA fragments of chlamydial MOMP genes of the psittacosis group and group-specific MOMP gene fragments of the trachoma, pneumonia, and polyarthritis groups were restricted. Appropriate restriction enzymes were identified by computer analysis of the partial MOMP gene DNA sequences (12). The MOMP genotypes of these chlamydial groups were differentiated as *ompA* RFLP types with the following restriction endonucleases: psittacosis with *Hae*III, trachoma with *Pvu*II, pneumonia with *Fnu*4HI, and polyarthritis with *Bst*UI and *Bst*YI. Ten to 20 μl of the secondary PCRs was mixed with the appropriate buffer and restricted for 1 h at the recommended temperature by using 10 U of the respective enzymes (New England Biolabs, Beverly, Mass.). The specificity of the amplification was confirmed through (i) the appearance of a single DNA fragment of the expected size and (ii) the appearance of restriction patterns of the amplified secondary DNA fragments as expected from the DNA sequences of the partial MOMP genes.

Clinical samples. Seven milk samples were obtained from cows suffering from chronic mastitis of variable severities. These cows were randomly selected from different herds surveyed in a mastitis control program. Low numbers of *E. coli* were found in sample 2200 LR by standard bacteriological techniques; all other samples were free of detectable bacteria. DNA was extracted as described earlier (13). Samples of 10 μl of DNA (0.2 to 0.7 μg) were subjected to primary amplification, and subsequent procedures were performed as described above.

RESULTS

Determination of the sensitivity of genus-specific PCR amplification. The efficiency of PCR amplification was optimized in a series of experiments (11). Subsequently, the sensitivities of primary and combined primary and secondary genus-specific amplifications were determined. The input DNA consisted of 10-fold dilutions of purified B577 DNA in a constant background of 1.0 μg of uninfected L-cell and bacterial DNAs (which is the equivalent of 3×10^5 human genomes or 3×10^8 *E. coli* genomes, respectively). The number of chlamydial templates was calculated by assuming that the MOMP gene is a single-copy gene (3) within the 1,450-kbp chlamydial genome (6) (1,563 ng of chlamydial DNA equals 10^6 templates).

Chlamydial DNA representing 10^5 chlamydial genomes (156.3 pg of DNA) yielded an expected single DNA fragment of approximately 1,120 bp after primary amplification (Fig. 2). This fragment was clearly visible after ethidium bromide staining. In contrast, DNA representing 10^4 chlamydial genomes produced a 1,120-bp DNA fragment that stained faintly in the original photograph but that was not intensive

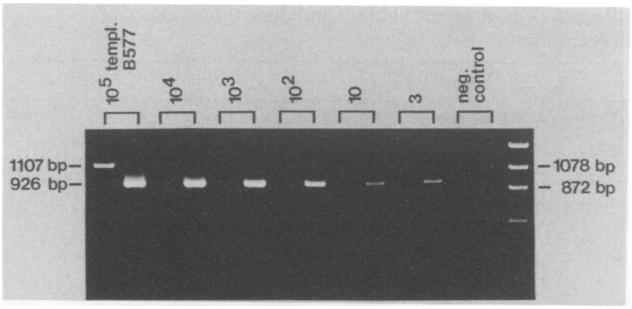

FIG. 2. Determination of the sensitivity of primary and combined primary and secondary genus-specific PCR amplifications of the B577 MOMP gene. The indicated amounts of purified B577 template (temp.) DNA were mixed with 1.0 μg of background DNA per 100-μl reaction and were analyzed by PCR as described in the text. DNA fragments were resolved by 1.5% agarose gel electrophoresis (1:1 ratio of DNA-grade agarose to NuSieve agarose) and ethidium bromide staining. Primary amplification products can be observed in the respective left lanes; secondary products can be observed in the respective right lanes; negative (neg.) control indicates background DNA without template. The molecular size marker was φX174RF DNA digested with *Hae*III.

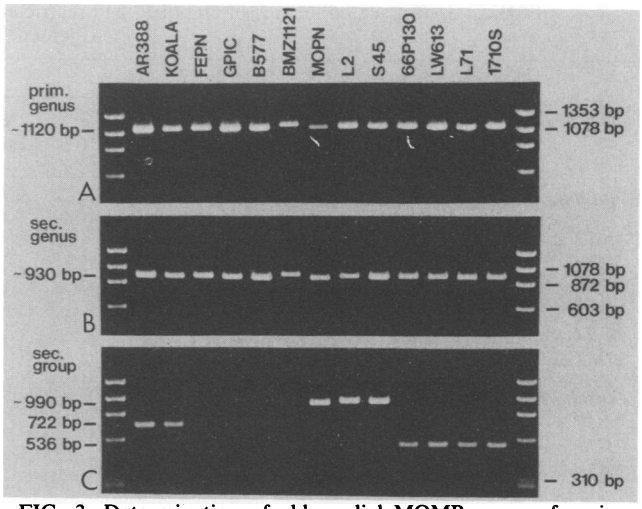

FIG. 3. Determination of chlamydial MOMP genes of various genotypes amplified in primary (A), secondary genus-specific (B), and secondary group-specific (C) PCRs. DNA extracted from yolk sacs infected with chlamydial strains with different MOMP genotypes or purified chlamydial DNA (AR388 and KOALA) was subjected to primary and secondary amplifications. AR388 and KOALA represent the pneumonia group; FEPN, GPIC, B577, and BMZ1121 represent the psittacosis group; MOPN, L2, and S45 represent the trachoma group; and 66P130, LW613, L71, and 1710S represent the polyarthritis group of MOMP genotypes. DNA fragments were resolved by 1.5% agarose gel electrophoresis and ethidium bromide staining.

enough for visible reproduction in Fig. 2. A single DNA fragment of the expected size of approximately 930 bp was observed in the secondary amplification. Chlamydial DNA representing three primary input genomes was sufficient for visualization of the secondary 930-bp DNA fragment after ethidium bromide staining.

Thus, combined primary and secondary genus-specific amplifications are capable of detecting one cognate chlamydial genome in a 2.10×10^8-fold excess of unrelated DNA or in a background of DNA derived from 10^5 human cells.

Genus-specific PCR amplification of chlamydial MOMP gene fragments. To verify the spectrum of MOMP genes of *Chlamydia* that can be amplified, we isolated DNA from yolk sacs infected with strains with representative chlamydial MOMP genotypes. Diluted samples (1:100) were subjected to primary and secondary amplifications. The results indicated that all chlamydial MOMP genes were amplified by the genus-specific oligonucleotide primers (Fig. 3A and B). Specific amplification of the chlamydial target DNA was indicated by the presence of a single specific DNA fragment in electrophoretic analysis of primary and secondary genus-specific PCRs. The slightly different migrations of DNA fragments indicated size differences in the MOMP genes.

Thus, the two-step PCR of the chlamydial MOMP gene with the primers 9CHOMP, CHOMP371, 29CHOMP, and CHOMP336 amplifies DNA fragments from the known chlamydiae and might also detect as yet undefined chlamydial variants.

Differentiation of groups of chlamydial MOMP genotypes by PCR amplification. The secondary group-specific PCR was developed as a tool for rapid preliminary classification of chlamydial MOMP genotypes. The antisense, group-specific primers TROMP358, PNOMP268, and PSOMP204 were designed to hybridize under stringent conditions with different fingerprint regions of the MOMP genes of their respective cognate groups but not with chlamydial MOMP genes of the other groups. This resulted in a multiplex PCR (4) in which the genus-specific sense primer 29CHOMP was shared by all amplified DNA fragments (see Fig. 3C and 5C).

Chlamydiae of the trachoma, pneumonia, and polyarthritis groups of MOMP genotypes yielded specific amplified DNA fragments of the expected lengths (Table 3). As anticipated, MOMP genes of strains FEPN, GPIC, B577, and BMZ1121 of the psittacosis group could not be amplified. This group was identified through positive amplification only in the secondary genus-specific PCR.

The positive control sample (see left lane of Fig. 5C) was the secondary group-specific amplification of a sample from a primary PCR containing DNA of chlamydial MOMP genotypes represented by strains L2, AR388, and LW613. The simultaneous appearance of DNA fragments specific for the chlamydial groups trachoma, pneumonia, and polyarthritis confirmed the potential of the secondary group-specific amplification to identify multiple infections with certain chlamydial MOMP genotypes.

Thus, in combination with genus-specific PCRs, the secondary group-specific amplification differentiated between four groups of chlamydial MOMP genotypes. Furthermore, it identified the simultaneous presence of chlamydial strains of the trachoma, pneumonia, and polyarthritis groups.

Differentiation of chlamydial MOMP genotypes by restriction endonuclease analysis of amplified DNA fragments. Knowledge of the partial MOMP gene DNA sequences of the chlamydial MOMP genotypes listed in Tables 1 and 3 enabled us to further differentiate them as *ompA* RFLP types by restriction endonuclease analysis of secondary group-specific or genus-specific (psittacosis group) amplified DNA fragments. Appropriate restriction endonucleases were rapidly identified by computer-assisted restriction mapping of the partial MOMP genes. These enzymes and the expected DNA fragment sizes after restriction are given in Table 3. The differentiation scheme was designed such that closely related MOMP genotypes represented by strains MN, 6BC, and OA of the psittacosis group were recognized as restriction fragments of the same length representing the *ompA*

TABLE 3. Calculated lengths of amplified and restricted MOMP gene DNA fragments of chlamydial strains included in PCR detection and differentiation[a]

Chlamydial strain	Chlamydial group	Length (bp)			
		Primary genus-specific PCR	Secondary genus-specific	Secondary group-specific PCR	Secondary PCR restriction analysis
C. pneumoniae AR388	Pneumonia	**1,104**	**923**	**722**	**576, 143**, 3 (*Fnu*4HI)
C. psittaci					
KOALA	Pneumonia	**1,104**	**923**	**722**	**362, 360** (*Fnu*4HI)
FEPN	Psittacosis	**1,116**	**935**		**796, 88**, 51 (*Hae*III)
GPIC	Psittacosis	**1,107**	**926**		**395, 201, 197, 133** (*Hae*III)
B577	Psittacosis	**1,107**	**926**		**926** (*Hae*III)
BMZ1121	Psittacosis	**1,146**	**965**		**710, 255** (*Hae*III)
C. trachomatis					
MOPN	Trachoma	**1,101**	**920**	**981**	**614, 367** (*Pvu*II)
L2	Trachoma	**1,122**	**941**	**1,002**	**565 437**, (*Pvu*II)
S45	Trachoma	**1,119**	**938**	**999**	**420, 379, 200** (*Pvu*II)
C. psittaci					
66P130	Polyarthritis	**1,116**	**935**	**536**	**468**, 37, 18, 13 (*Bst*UI and *Bst*YI)
LW613	Polyarthritis	**1,110**	**929**	**536**	**294, 105**, 69, 37, 18, 13 (*Byt*UI and *Bst*YI)
L71	Polyarthritis	**1,101**	**920**	**536**	**294, 174**, 37, 18, 13 (*Bst*UI and *Bst*YI)
1710S	Polyarthritis	**1,104**	**923**	**536**	**399**, 69, 37, 18, 13 (*Bst*UI and *Bst*YI)

[a] Oligonucleotide primers 9CHOMP and CHOMP371 were used in genus-specific primary amplification; 29CHOMP and CHOMP336 were used in genus-specific secondary amplification. Primers 29CHOMP, TROMP358, PNOMP268, and PSOMP204 were used in secondary group-specific amplification. For restriction endonuclease analysis, the amplified secondary group-specific PCR fragments of trachoma, pneumonia, and polyarthritis groups of MOMP genotypes and secondary genus-specific PCR fragments of psittacosis group MOMP genotypes were digested with the indicated restriction enzymes. The DNA fragments that were visible in agarose gels are indicated in boldface type. The fragment lengths were calculated by using published MOMP gene DNA sequences (1, 12, 24, 28) and additional unpublished data deposited in GenBank (accession codes M73033 to M73040, and M73042 to M73044).

RFLP type MN and types B577 and LW508 as another identical *ompA* RFLP type B577 (12). Differentiation of the trachoma group MOMP genotypes represented by strains S45, MOPN, and L2 with *Pvu*II is not comprehensive for all human serovars of *C. trachomatis* and was intended to merely discriminate between the given set of amplified MOMP genotypes of the trachoma group.

Figure 4 demonstrates that cleavage with the respective restriction endonucleases generated DNA fragments of the expected size. Some fragments exhibited slightly aberrant mobilities, such as the 395-bp DNA fragment of strain GPIC (apparent mobility, approximately 360 bp) and, most notably, the 576-bp fragment of strain AR388, which migrated with an apparent mobility of approximately 620 bp. These aberrant mobilities were consistently found in analyses with different agarose concentrations and electrophoresis buffers. However, the original secondary DNA fragments of the MOMP genes of the different genotypes represented by these strains had the correct lengths (Fig. 3B and C), and after restriction the remaining fragments of the original amplified DNAs were identified at the correct positions.

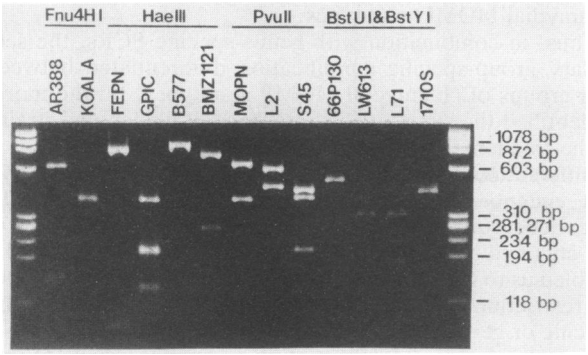

FIG. 4. Differentiation of *ompA* RFLP types by restriction endonuclease analysis of secondary amplification products. Ten to 20 μl of amplified secondary PCRs (genus specific for the psittacosis group; group specific for the trachoma, pneumonia, and polyarthritis groups) was mixed with restriction buffer and incubated with 10 U of the indicated restriction endonucleases. DNA fragments were resolved by 4% agarose gel electrophoresis (1:3 ratio of DNA-grade agarose to NuSieve agarose) and ethidium bromide staining.

These results indicate that restriction endonuclease analysis of secondary MOMP gene amplification products can unambiguously achieve the intended differentiation of chlamydial *ompA* RFLP types.

PCR analysis of clinical samples. We analyzed the milk of seven dairy cows suffering from mastitis to assess the applicability of the established PCR amplification for detection of chlamydial MOMP gene sequences in clinical samples. Specific DNA fragments could not be visualized in the primary amplification, indicating that there were a low number of chlamydiae in the samples (Fig. 5). Secondary genus-specific amplification yielded strongly staining, specific DNA fragments in samples 2200 LR and 2361 LF in repeat examinations, while all other samples remained negative. Secondary group-specific amplifications were negative in all samples, indicating that the MOMP gene fragments amplified in the secondary genus-specific PCRs were derived from the psittacosis group of chlamydial MOMP genotypes. DNA fragments of these reactions were therefore analyzed by restriction with *Hae*III. The approximately 1,120-bp fragments obtained after restriction indicated that the *C. psittaci*-positive milk samples contained MOMP gene sequences of the *ompA* RFLP type B577.

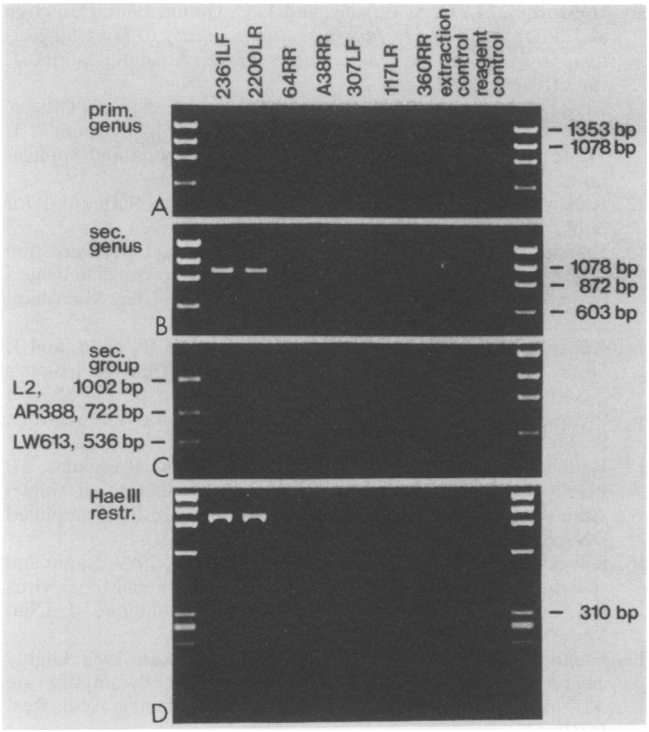

FIG. 5. PCR analysis of milk from seven cases of bovine mastitis. DNA extracted from milk samples were subjected to primary (A), secondary genus-specific (B), and secondary group-specific (C) amplifications. DNA fragments from secondary genus-specific PCRs of samples 2361 LF and 2200 LR were analyzed by restriction with *Hae*III (D). The extraction control was performed with a mock-extracted sample; the reagent control was a 100-μl PCR in the absence of a DNA sample. DNA fragments were resolved by 1.5 or 4% agarose gel electrophoresis and ethidium bromide staining. PCR products and *Hae*III restriction products are indicated on the left side of the respective panels. A secondary group-specific PCR is included as a positive control in the leftmost lane of panel C. The primary input sample of this PCR contained DNA of chlamydial MOMP genes of genotypes L2 (trachoma group), AR388 (pneumonia group), and LW613 (polyarthritis group).

These results demonstrate that the *Chlamydia* genus-specific PCR amplification can be successfully applied to clinical samples and that the *ompA* RFLP type contained in positive samples can be determined by restriction endonuclease analysis of secondary amplification products.

DISCUSSION

A two-step PCR amplification procedure with a nested set of degenerate oligonucleotide primers specific for the MOMP gene of the genus *Chlamydia* was developed. This method can detect and differentiate chlamydial MOMP gene DNA fragments by a combination of group-specific amplification and RFLP analysis. The detection limit of this method approached three chlamydial templates in the presence of 1 μg of eucaryotic and bacterial background DNAs. We performed 27 secondary PCR cycles, resulting in a sensitivity limit of three chlamydial templates, because, occasionally, the negative control reactions became weakly positive when additional cycles of secondary amplification were used. Thus, the two-step PCR amplification procedure offers the advantage that the sensitivity of the system can easily be adjusted.

The absolute detection limit of this method is given by the ratio of chlamydial target DNA to background DNA. The minimal ratio is one target molecule per approximately 1 μg of DNA in a 100-μl PCR. At background DNA levels above 1 μg per 100-μl PCR, unspecific amplification dramatically increases while the efficiency of the specific amplification is reduced. Consequently, the sensitivity of chlamydial detection can be further improved only by minimizing the amount of background DNA. This can be achieved by differential sedimentation of cellular nuclei after sonication of a sample.

Assuming a Poisson distribution in DNA samples, false-negative results are predicted to appear with low amounts of chlamydial DNA. Analysis of multiple samples are necessary to confirm truly negative reactions.

An important advantage of the two-step PCR detection procedure with two nested pairs of unrelated primers is its high specificity (16). This method requires only minimal laboratory equipment for amplification and detection of the amplified DNA fragments. It can be performed in 1 workday without additional detection techniques, such as Southern hybridization or enzyme-linked immunosorbent assay-based hybrid capture assays (2).

We designed the genus-specific primers 9CHOMP, CHOMP371, 29CHOMP, and CHOMP336 from highly conserved DNA sequences of the 5′ and 3′ ends of the translated *ompA* regions for recognition of the broadest possible spectrum of chlamydial MOMP genes. The chlamydial strains analyzed in this investigation constitute a comprehensive set of chlamydial variants. These primers matched the DNA sequences of genes of MOMP genotypes not examined in this study (those of strains 6BC, OA, LW508) and some serovars of *C. trachomatis* except L2. The complete MOMP gene DNA sequences of *C. trachomatis* serovars Ba, D, G, I, J, K, and L3 have not been determined. The cognate regions of the primers at the 5′ and 3′ ends of the MOMP gene are the most highly conserved *ompA* sequences. It is expected that these primers will hybridize and amplify these genes.

The secondary group-specific amplification facilitated rapid identification of the overall position within the chlamydial phylogeny of an amplified MOMP gene DNA fragment. It was also capable of detecting mixed infections with chlamydial strains of the trachoma, pneumonia, and polyarthritis groups. The sensitivity of this amplification varied slightly, depending on the length of the amplified group-specific DNA fragment, but it could be adjusted like the secondary genus-specific amplification could.

Further differentiation of amplified products can be achieved by RFLP analysis. The availability of MOMP gene DNA sequences allows an appropriate selection of restriction endonucleases for RFLP analysis.

While the RFLP analysis of the psittacosis, polyarthritis, and pneumonia groups differentiated a spectrum of MOMP genotypes, the analysis of the trachoma group was not comprehensive. The complete MOMP gene DNA sequences of *C. trachomatis* serovars Ba, D, G, I, J, K, and L3 are needed for complete RFLP typing of the trachoma group.

The detection of MOMP gene DNA sequences in two of seven milk samples from cases of bovine mastitis and the subsequent differentiation of the secondary genus-specific DNA fragments by *Hae*III restriction enzyme analysis demonstrate the feasibility of PCR-based detection and differentiation of chlamydiae in clinical samples. It will be necessary to optimize the extraction of DNA from the sample and the handling of large numbers of samples for PCR detection of chlamydiae in the clinical setting.

The data presented in this report indicate that the MOMP gene of extant variants of the genus *Chlamydia* can be detected by a two-step PCR. Amplified DNA fragments can be easily differentiated by group-specific secondary amplification and RFLP analysis. This differentiation combined with rapid DNA sequencing of secondary genus-specific amplification products (12, 15) could lead to the identification of as yet undefined chlamydial variants. Unlike chlamydial infections in humans, the diseases caused by chlamydiae in animals are not widely recognized. We anticipate that PCR-based genus-specific detection and differentiation of *Chlamydia* spp. will contribute to understanding of the epidemiology and pathogenesis of chlamydial infections.

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