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B. Kaltenboeck
School of Veterinary Medicine

K. G. Kousoulas
School of Veterinary Medicine

J. Storz
School of Veterinary Medicine

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Detection and Strain Differentiation of *Chlamydia psittaci* Mediated by a Two-Step Polymerase Chain Reaction

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

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Specific and sensitive amplification of major outer membrane protein (MOMP) gene DNA sequences of *Chlamydia psittaci* was achieved in a two-step polymerase chain reaction. First, oligonucleotide primers specific for 5′ and 3′ nontranslated regulatory regions of the MOMP gene were used in a polymerase chain reaction to amplify a DNA fragment of approximately 1,400 bp. A portion of this DNA fragment was amplified in a second reaction using a degenerate oligonucleotide primer specific for a DNA sequence contained within the 1,400-bp DNA fragment and one of the first-step primers. This method detected 10 cognate chlamydial genomes. *C. psittaci* MOMP genes from two avian strains and from mammalian serovars 1, 7, and 8 were amplified and analyzed by restriction endonuclease digestion. MOMP genes from mammalian serovars 2 through 6 and 9 and from strains of *C. trachomatis* and *C. pneumoniae* could not be amplified. Restriction endonuclease analysis with *Hae*III indicated a close relationship between *C. psittaci* strains of avian and mammalian serovar 1 lineage, while those of mammalian serovars 7 and 8 exhibited distinct restriction patterns. DNA sequences corresponding to the mammalian serovar 1–wild type parakeet MOMP genotype of *C. psittaci* were detected in two of seven milk samples from cases of bovine mastitis.

Infections with *Chlamydia psittaci* have a surprisingly wide distribution in the animal kingdom. Chlamydial infections may lead to overt clinical diseases, such as enteritis and placental and fetal infections, with abortion, sterility, pneumonia, polyarthritis, encephalitis, and mastitis, and cause significant losses in the animal industries. This includes poultry, in which these infections cause air sacculitis and death. Frequently, chlamydial infections persist in animals as clinically inapparent stages, with shedding of the pathogen over long periods (22).

Diagnosis of chlamydial infections in animals still represents a considerable challenge (22). Serological diagnosis is useful only retrospectively following overt disease. Firm evidence of the presence of chlamydiae is required for unequivocal diagnosis because of the preponderance of clinically inapparent and persistent infections. Isolation in cell culture remains difficult and depends on the presence of sufficient numbers of viable, infectious elementary bodies (EB). Sensitive methods for chlamydial detection which are not based on infectivity are needed to identify *C. psittaci*, particularly in persistent infections. It was the goal of this study to develop a sensitive method for detection of *C. psittaci*. In addition, detection and differentiation of this highly heterogeneous species is needed for epidemiological applications.

One approach to the problem was the development of enzyme-linked immunosorbent assay methods for detection of *C. psittaci* antigens. Antibodies to genus- or species-specific antigenic determinants of chlamydiae, which would provide a broad diagnostic spectrum, react weakly with chlamydial EB because these epitopes are not readily accessible to antibody (14). Thus, the sensitivity of enzyme-linked immunosorbent assay traps developed for detection of *C. trachomatis* surface antigens has remained problematic. Immunological detection of genus-specific epitopes on lipopolysaccharide is used as an alternative to cell culture isolation. Reports are controversial, and sensitivity generally does not exceed that of cell culture isolation (15, 23, 24). Furthermore, chlamydial species or serovars cannot be distinguished with this method.

The recently introduced technique of the polymerase chain reaction (PCR) increases the sensitivity and specificity of detection of pathogenic microorganisms by amplifying target DNA molecules by a factor of up to 10^6 (20). An advantage of nucleic acid-based detection of a pathogen is the relatively high stability of DNA without the need for transcription and translation. Also, production of a large amount of the targeted DNA fragment permits restriction endonuclease mapping of polymorphisms of the amplification product, thus facilitating differentiation.

The major outer membrane protein (MOMP) is the major structural protein exposed on the surface of the infectious EB (1). Species-, subspecies-, and serovar-specific epitopes are associated with the MOMP of *C. trachomatis* and conform to serovars as determined by microimmunofluorescence (29). Similarly, avian and mammalian strains of *C. psittaci* were differentiated by the reactivity of the MOMP with polyclonal antisera (1). The nucleic acid sequences of the MOMP genes of all serovars of *C. trachomatis* and of *C. psittaci* strains from guinea pig inclusion conjunctivitis (GPIC), meningopneumonitis, and ovine abortion contain four variable domains (VD) interspersed within highly conserved regions (7, 8, 18, 19, 21, 28, 29). VDI, VDII, and VDIV contain the known immunodominant and surface-exposed epitopes of infectious EB. MOMP genes of *C. psittaci* and *C. trachomatis* exhibit 68% DNA sequence homology. In contrast, the overall DNA homology between these two chlamydial species is less than 10%.

The MOMP gene was selected for these reasons as the target for PCR-based detection and differentiation of *C. psittaci*. Primers derived from DNA sequences of 5′ and 3′

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

TABLE 1. Origins, biovars, and serovars of *C. psittaci* strains included in the PCR analysis of chlamydial MOMP genes

Chlamydial strain	Biovar	Serovar	Host	Host clinical condition
B-577	1	1	Sheep	Abortion
BM-Z1121	2	ND[a]	Cow	Mastitis
LW-613	2	2	Calf	Polyarthritis
66-P-130	3	3	Calf	Normal (feces)
L-71	4	4	Swine	Polyarthritis
S-45	5	5	Swine	Normal (feces)
1710-S	4	6	Swine	Abortion
Fe-Pn	7	7	Cat	Live vaccine
GPIC	8	8	Guinea pig	Conjunctivitis
JP-751	ND	9	Sheep	Normal (feces)
6BC	ND	ND	Parakeet	Septicemia
wt parakeet	ND	ND	Parakeet	Septicemia

[a] ND, not determined.

nontranslated regulatory regions of the MOMP gene were selected because (i) these regions are conserved in all known sequences of *C. psittaci* MOMP genes but are different in *C. trachomatis*, thus undesired amplification is avoided, and (ii) amplification of the complete MOMP gene of *C. psittaci* provides maximum potential for subsequent differentiation of the amplification products.

This report describes a two-step PCR method for sensitive detection of MOMP gene sequences from some serovars of *C. psittaci*. Milk samples from dairy cows with mastitis were also tested with this method. Furthermore, we present evidence that differentiation of *C. psittaci* can be achieved by analysis of restriction fragment length polymorphism of the amplified DNA fragments.

(This report contains parts of a dissertation presented by B.K. to the Graduate School of Louisiana State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.)

MATERIALS AND METHODS

Chlamydial strains. Ten strains of *C. psittaci* representing nine mammalian serovars and six biovars of bovine, ovine, porcine, feline, and guinea pig origin (17) and two avian strains were used in this investigation. Relevant information about these isolates is summarized in Table 1. All strains of *C. psittaci* and *C. trachomatis* LGV-2 and Mo-Pn (mouse pneumonitis) were propagated in developing chicken embryos as described previously (17). In addition, strain B-577 was also cultivated in persistently infected L cells (16).

Extraction of chlamydial DNA. Heavily infected yolk sacs or cultured cells were homogenized in 2 ml of sucrose-phosphate buffer. The suspension was centrifuged at 200 × *g* for 10 min at 4°C (22). The chlamydiae present in the supernatant were sedimented at 14,000 × *g* for 30 min at 4°C. Sediments from infected yolk sacs were directly subjected to DNA extraction. Chlamydial harvests from cell cultures were further processed to obtain purified chlamydial DNA. The pellet was dissolved in DNase buffer (10 mM Tris-HCl [pH 7.2], 10 mM $MgCl_2$) and incubated at 37°C for 30 min with 100 μg of pancreatic DNase A (Sigma, St. Louis, Mo.) per ml. This suspension was layered onto a Renografin (E. R. Squibb & Sons, Princeton, N.J.) step gradient (32, 44, 54, and 60%) in 20 mM Tris-HCl (pH 7.5)–150 mM KCl and centrifuged at 90,000 × *g* for 2 h at 4°C (25). EB at the 44 to 54% interface were washed twice in 38 ml of sucrose-phosphate buffer and sedimented at 14,000 × *g* for 30 min for

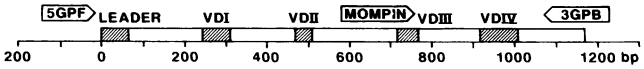

FIG. 1. Schematic representation of the GPIC MOMP gene with the primers for primary (5GPF and 3GPB) and secondary (MOMPIN and 3GPB) PCRs. Translated regions and primers are boxed. VD encoding antigenic determinants are interspersed among highly conserved regions. Primers are drawn to scale only at their 5′ ends.

DNA isolation by cetyltrimethylammonium bromide extraction (27). Briefly, EB were suspended in TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA), sodium dodecyl sulfate was added to 0.5%, and proteinase K was added to 100 μg/ml, and the suspension was incubated for 1 h at 37°C. NaCl was then added to 0.7 M, and cetyltrimethylammonium bromide was added to 1%. DNA was extracted with chloroform and phenol-chloroform and precipitated by addition of 0.6 volume of isopropanol. The precipitate was washed with 70% ethanol and dissolved in 100 μl of TE. RNA was removed from purified B-577 DNA by RNase A (100 μg/ml) digestion (30 min, 37°C), followed by phenol-chloroform extraction. Purified DNA from *C. pneumoniae* AR-388 was a kind gift of L. A. Campbell, University of Washington, Seattle.

Chlamydia-negative background DNAs from uninfected L cells and bacteria (*Escherichia coli*, *Salmonella choleraesuis*, *Bordetella bronchiseptica*, and *Pseudomonas aeruginosa*) were extracted after low-speed sedimentation as described above. Chlamydiae do not possess a closely related eubacterial genus (26), which is more likely than other microorganisms to cause unspecific amplification. This DNA was included in the amplification background because such types of pathogenic microorganisms are likely to be encountered in clinical specimens screened for the presence of *C. psittaci*.

Southern blots of purified B-577 and L-cell DNAs were probed with digoxigenin (Boehringer Mannheim, Indianapolis, Ind.)-labeled, purified B-577 DNA to evaluate the purity of chlamydial DNA. While the labeled B-577 DNA strongly hybridized to blotted B-577 DNA, no signal was observed with blotted L-cell DNA, indicating that no L-cell DNA was present in the labeled, purified B-577 DNA.

Oligonucleotide primers. Oligonucleotide primers were obtained from a commercial source (Genetic Designs, Inc., Houston, Tex.) and used without further purification. Primers 5GPF (5′-ACGCATGCAAGACACTCCTCAAAGCC-3′) and 3GPB (5′-ACGAATTCCTAGGTTCTGATAGCGGG AC-3′) were derived from MOMP gene DNA sequences of strains of *C. psittaci* (8, 18, 29). They are identical to bases −140 to −120 of the 5′ nontranslated region and complementary to bases +50 to +30 of the 3′ nontranslated region of the GPIC MOMP gene. *Sph*I and *Eco*RI restriction sites were included within the 5′ ends of 5GPF and 3GPB, respectively, to facilitate cloning. The 32-fold degenerate, inosine containing primer MOMPIN (5′-GCI[CT]T[CI]TGGGAITG[CT]GG [CI]TG[CT]GCIAC3′) was designed to hybridize to the noncoding strand of a chlamydial MOMP gene sequence coding for conserved amino acids 173 to 181 of the GPIC MOMP (13). A scheme of the MOMP gene and primers is depicted in Fig. 1.

PCR amplification of chlamydial DNA. PCR was performed with 2.5 U of *Taq* DNA polymerase (GIBCO-BRL, Gaithersburg, Md.) in 100-μl reactions containing 0.01% gelatin and 0.05% Tween 20 in 10 mM Tris-HCl (pH 8.3)–1.5 mM $MgCl_2$–50 mM KCl–each deoxynucleoside triphosphate

(dNTP) at 200 μM–each primer (5GPF and 3GPB in primary amplification and MOMPIN and 3GPB in secondary amplification) at 0.2 μM. To avoid PCR product carryover, positive-displacement pipettes were used, 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 subjected to 40 cycles of 1 min at 94°C, 1 min at 59°C, and 2 min at 72°C in primary amplification and 25 cycles in secondary amplification in a programmable DNA thermal cycler (Perkin Elmer-Cetus, Norwalk, Conn.). The sensitivity of the PCR was established by using various amounts of purified B-577 DNA in a background of 0.7 μg of a mixture of uninfected L-cell and bacterial DNAs as the input for primary amplification. Samples (1 μl) of the amplified primary reaction were used as the input DNA in the secondary PCR. Negative controls with and without background DNA (extraction and reagent controls) were routinely included. To establish the spectrum of chlamydial strains with DNA that can be amplified, 10 μl of 1:100-diluted DNA extracted from infected yolk sacs (30 to 100 ng) was used in the primary PCR.

Analysis of amplified DNA. Samples (10 μl) of the amplified reaction were fractionated by 1.5% agarose gel electrophoresis directly or after restriction endonuclease digestion. The DNA was visualized by ethidium bromide staining and UV fluorescence. PCR mixtures with the appropriate buffer were restricted for 1 h at 37°C by using 10 U of the respective enzyme. The specificity of the amplified primary DNA fragment was confirmed through (i) appearance of a single or predominant fragment of the expected size in secondary amplification using 3GPB and the internal primer MOMPIN, (ii) appearance of a *Hae*III restriction pattern of the amplified GPIC fragment as expected from the DNA sequence of the GPIC MOMP gene (29), and (iii) comparison of a partial B-577 MOMP gene DNA sequence with known MOMP gene sequences of strains of *C. psittaci*.

Asymmetric PCR was used to prepare single-stranded template DNA for partial dideoxy DNA sequencing of the B-577 MOMP gene (6). The conditions used were 25 cycles with each dNTP at 20 μM, 5GPF at 0.02 μM, 3GPB at 0.25 μM, and 10 μl of the 10^{-3}-diluted primary PCR product, and gelatin was omitted from the reaction buffer. dNTPs and primers were removed by spin dialysis with Centricon-100 devices (Amicon, Danvers, Mass.), and 7 μl of the dialysate was used for sequencing by the dideoxy-chain termination method using T7 DNA polymerase as recommended by the manufacturer (United States Biochemicals, Cleveland, Ohio). Ten picomoles of 5GPF or MOMPIN was used as the primer, and [^{35}S]dATP-labeled reaction products were separated by 6% polyacrylamide–urea gel electrophoresis and visualized by autoradiography.

Clinical samples. Seven milk samples were obtained from cows with chronic mastitis of various degrees of severity. These cows were randomly selected from different herds surveyed in a mastitis control program. Low numbers of *E. coli* organisms were found in sample 2200 LR by standard bacteriological techniques, and all other samples were free of detectable bacteria. DNA was extracted as described for infected yolk sacs, except that homogenization was performed without adding sucrose-phosphate buffer. DNA (0.2 to 0.7 μg) was subjected to primary amplification, and subsequent procedures were performed as already described.

Samples positive for *C. psittaci* in the secondary amplification, but with no visible primary amplification product, were further processed for restriction analysis of the primary fragment. A 90-μl volume of the primary reaction was subjected to spin dialysis with Centricon-100 devices, and 10 μl of the dialysate was reamplified for 25 cycles with primers 5GPF and 3GPB. These reactions were analyzed by *Hae*III digestion and agarose gel electrophoresis.

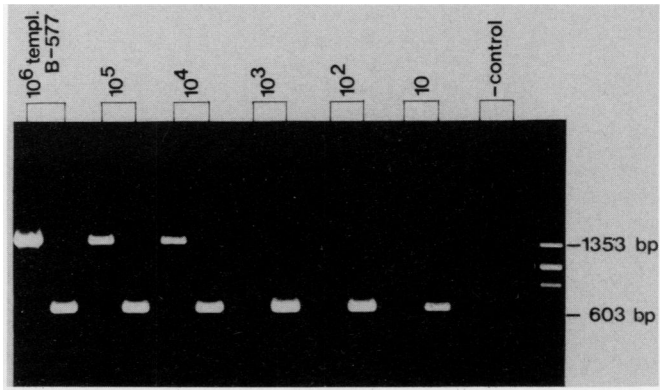

FIG. 2. Determination of the sensitivities of primary and combined primary and secondary PCR amplifications of B-577 MOMP gene sequences. The indicated amounts of purified B-577 template (templ.) DNA were mixed with 0.7 μg of background DNA per 100-μl reaction and subjected to thermocycling as described in Materials and Methods. Fragments were resolved by 1.5% agarose gel electrophoresis and ethidium bromide staining. Primary amplification products can be observed in the respective left-hand lanes, and secondary products are in the right-hand lanes. Control indicates background DNA with no template. The molecular weight marker was ϕX174RF DNA digested with *Hae*III.

RESULTS

PCR optimization. The efficiency of amplification was optimized in a series of experiments (10). The parameters found to be most critical were the primer concentration of 0.2 μM, the stringent annealing temperature of 59°C, a maximum of 1 μg of input DNA per 100-μl reaction, and the dNTP concentration of 200 μM. At Mg^{2+} concentrations above 3 mM, the efficiency of target amplification was reduced and aberrant DNA fragments appeared.

Determination of the sensitivity of PCR amplification. Once optimal conditions were established, the sensitivities of primary and combined primary and secondary amplifications were determined. The input DNA consisted of 10-fold dilutions of purified B-577 DNA in a constant background of 0.7 μg of uninfected L-cell and bacterial DNAs (equivalent of 2.1×10^5 human genomes or 2.1×10^8 *E. coli* genomes, respectively). The number of chlamydial templates was calculated by assuming that the MOMP gene is a single-copy gene (2) within the 1,450-kbp chlamydial genome (4) (1.563 ng of chlamydial DNA equals 10^6 templates).

Chlamydial DNA representing 10,000 chlamydial genomes (15.63 pg of DNA) yielded an expected single DNA fragment of approximately 1,400 bp after primary amplification. This fragment was clearly visible after ethidium bromide staining. In contrast, DNA representing 1,000 chlamydial genomes produced a faintly staining 1,400-bp DNA fragment which was visible in the original photograph but was too faint for visible reproduction in Fig. 2. In the secondary amplification, a single DNA fragment of the expected size of approximately 640 bp was observed. Chlamydial DNA representing 10 genomes was sufficient for visualization of the 640-bp

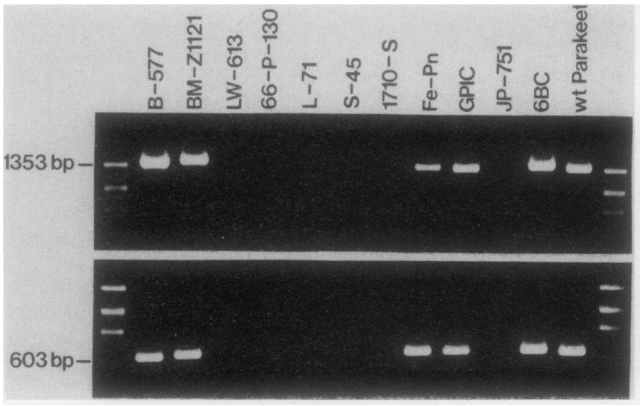

FIG. 3. Determination of strains of *C. psittaci* with MOMP genotypes amplified by primers 5GPF, 3GPB, and MOMPIN. DNAs extracted from yolk sacs infected with prototype strains of mammalian serovars and avian isolates (6BC and wt parakeet) of *C. psittaci* were subjected to primary and secondary amplifications. Fragments were resolved by 1.5% agarose gel electrophoresis and ethidium bromide staining. Primary amplification products can be observed in the upper panel, and secondary products are in the lower panel.

DNA fragment after ethidium bromide staining. Thus, combined primary and secondary amplifications were capable of detecting one cognate chlamydial genome in a 44-millionfold excess of unrelated DNA or in a background of DNA derived from 21,000 human cells.

Specificity of amplification determined by partial sequence analysis. The presence of a specific single or strongly predominant DNA fragment in electrophoretic analysis of primary and secondary PCRs in this investigation indicates amplification of the chlamydial target DNA (Fig. 2 and 3). Additional evidence for specific amplification is furnished by *Hae*III restriction of the GPIC primary amplification product. This restriction yielded DNA fragments of approximately 650, 300, and 200 bp (Fig. 4), which were expected from the known GPIC MOMP DNA sequence (29).

The most stringent proof of specificity of an amplification is the sequence comparison of the amplified fragment with a known target sequence. We established an asymmetric PCR with the external primers 5GPF and 3GPB to generate single-stranded DNA for sequencing of amplified fragments (6). A partial sequence of the B-577 primary DNA fragment was obtained by using 5GPF and MOMPIN as sequencing primers. This DNA sequence is identical to bases −55 to +90 and to constant-region bases +653 to +723 of the MOMP gene of ovine abortion isolate S26/3 of *C. psittaci* (8). This identity indicates that specific PCR amplification of the targeted chlamydial MOMP gene was achieved.

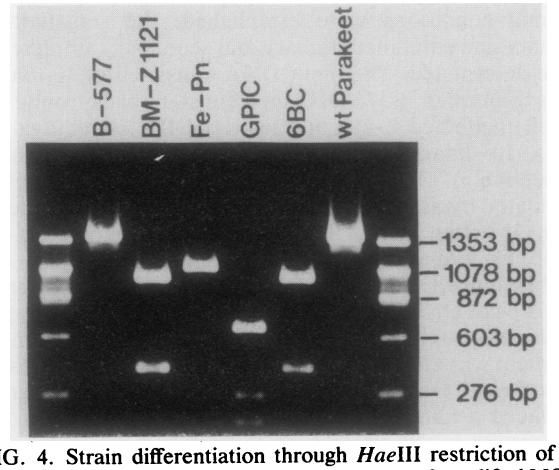

FIG. 4. Strain differentiation through *Hae*III restriction of amplified *C. psittaci* MOMP genes. A 10-μl volume of amplified MOMP gene DNA fragments was mixed with restriction buffer and digested with 10 U of *Hae*III. Fragments were resolved by 1.5% agarose gel electrophoresis and ethidium bromide staining.

PCR amplification is *C. psittaci* subspecies specific. To determine which MOMP genes of *C. psittaci* can be amplified, we isolated DNAs from yolk sacs infected with representative strains of different *C. psittaci* serovars. Diluted samples (1:100) were subjected to primary and secondary amplifications. The results indicate that MOMP genes of avian strains 6BC and wild-type (wt) parakeet; mammalian serovar 1 (B-577), 7 (Fe-Pn = Feline Pneumonitis), and 8 (GPIC) strains; and the serologically undefined isolate BM-Z1121 from bovine mastitis were amplified by the primers but strains from serovars 2 through 6 and 9 were not recognized (Fig. 3). The slightly different migration of DNA fragments indicates size differences in the MOMP genes. The MOMP genes of *C. trachomatis* LGV-2 and Mo-Pn and *C. pneumoniae* AR-388 were not detected (data not shown). Thus, two-step PCR detection of the chlamydial MOMP gene using primers 5GPF, 3GPB, and MOMPIN is specific for strains of the serovar 1, 7, and 8 subset of mammalian serovars of *C. psittaci* and for the subset of avian *C. psittaci* strains used in this study.

Strain differentiation by restriction endonuclease analysis of amplified fragments. We attempted to differentiate the chlamydial strains with amplifiable MOMP genes on the basis of the restriction fragment pattern. A computer search of the known MOMP gene sequences of *C. psittaci* revealed only a few restriction endonucleases with potential for restriction fragment length polymorphism differentiation. Restriction of the amplifiable MOMP genes with *Hae*III produced different DNA fragments (Fig. 4). *Hae*III restriction sites were not present in the B-577 and wt parakeet MOMP genes, as indicated by the full length amplification product of approximately 1,390 bp. In strains BM-Z1121 and 6BC, one apparently identical *Hae*III site was found which generated fragments of approximately 980 and 420 bp. *Hae*III restriction of the GPIC MOMP gene revealed four sites as expected from the sequence data. The calculated DNA fragment sizes are 643, 301, 201, 197, and 30 bp, which closely match the bands observed. Double molarity of the approximately 200-bp band was indicated by equally intensive staining compared with the 301-bp DNA fragment. The predicted 30-bp DNA fragment could not be identified in the 1.5% agarose gel. Serovar 7, comprising Fe-Pn of *C. psittaci*, yielded a PCR product with an apparently single *Hae*III site generating fragments of approximately 1,100 and 260 bp. This restriction pattern was easily differentiated from the restriction pattern in BM-Z1121 and 6BC (980 and 420 bp).

PCR analysis of clinical samples. We analyzed the milk of seven dairy cows with mastitis to assess the applicability of the established PCR amplification for detection of MOMP gene sequences of *C. psittaci* in clinical samples. Specific DNA fragments could not be visualized in primary amplification, indicating a low number of chlamydiae in the samples. Secondary amplification yielded a strongly staining, specific DNA fragment in samples 2200 LR and 2361 LF, while the rest remained negative (Fig. 5). Primary reactions of the positive samples were depleted of dNTPs, primers,

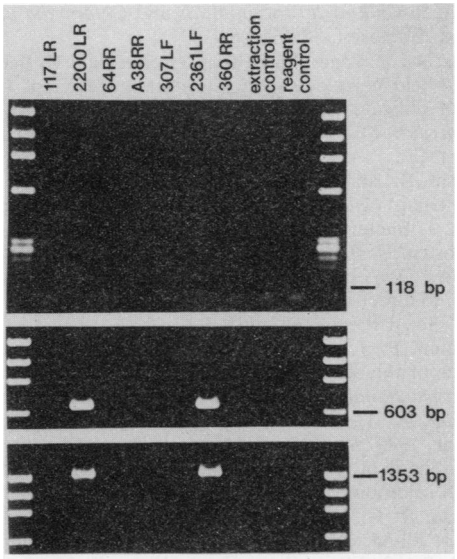

FIG. 5. PCR analysis of milk from seven cases of bovine mastitis. DNAs extracted from milk samples were subjected to primary and secondary amplifications. Primary reactions of positive samples were purified by centrifugal dialysis and reamplified with 5GPF and 3GPB. These DNA fragments were analyzed by *Hae*III restriction. Extraction control was performed with an extracted mock sample, and the reagent control was a 100-μl PCR with no sample added. Fragments were resolved by 1.5% agarose gel electrophoresis and ethidium bromide staining. Primary amplification products can be observed in the upper panel, secondary products are in the middle panel, and *Hae*III-restricted primary fragments of positive samples are in the lower panel.

and unspecific, low-molecular-weight PCR products (primer dimer) by spin dialysis and reamplified with 5GPF and 3GPB. DNA fragments of these reactions were analyzed by *Hae*III restriction. The approximately 1,390-bp fragments left after restriction indicate that the *C. psittaci*-positive milk samples contain MOMP gene sequences of the mammalian serovar 1-wt parakeet MOMP genotype. These results demonstrate that the *C. psittaci* subspecies-specific PCR amplification can be successfully applied to clinical samples. Furthermore, the MOMP genotype of the amplified fragment can be determined by *Hae*III restriction, facilitating the differentiation of MOMP genes of *C. psittaci* in conjunction with detection.

DISCUSSION

We have developed a two-step PCR amplification of MOMP genes of *C. psittaci*. Combined with restriction fragment length polymorphism of the amplified MOMP genes, this method can detect and differentiate several strains of *C. psittaci*. While an input of 1,000 target MOMP genes probably represents an absolute limit for direct visualization after a single amplification, the strong secondary DNA fragment, even with only 10 primary targets, indicates that a higher sensitivity of detection can be achieved. Careful titration of the visualization limit of the secondary amplification product considering a Poisson distribution at low target numbers would reveal the true detection limit of combined primary and secondary amplifications, which might be as much as 10-fold higher (1 genome equivalent per 0.7 μg of background DNA).

The detection of MOMP gene sequences in two of seven milk samples from bovine mastitis and the subsequent *Hae*III differentiation of these MOMP gene fragments demonstrate the feasibility of PCR diagnosis and differentiation of *C. psittaci* in clinical samples. We did not notice any differences in estimated amplification efficiencies among DNA from milk samples, chlamydia-infected yolk sac-derived DNA, or purified chlamydial DNA. The sensitivity of chlamydial detection in clinical samples should be equal to the results in this investigation. Differential centrifugation of the native sample might be important in certain cases for enriching chlamydial EB, as well as removing erythrocytes, because heme inhibits *Taq* DNA polymerase (9).

Mammalian serovars 1, 7, and 8 and two avian strains of *C. psittaci* could be amplified, but mammalian serovars 2 through 6 and 9, *C. trachomatis* Mo-Pn and LGV-2, and *C. pneumoniae* AR-388 were refractory. MOMP gene sequences of 5′ and 3′ nontranslated regulatory regions, targeted by primers 5GPF and 3GPB, of *C. trachomatis* A, B, C, F, H, LGV-1, and LGV-2 are highly conserved among these strains yet strongly divergent from *C. psittaci* sequences (7, 8, 18, 19, 21, 28, 29). Considering the overall extreme similarity between human *C. trachomatis* strains (3, 5, 12), we assume that in addition to LGV-2, other human strains of *C. trachomatis* cannot be detected with these primers. Comparisons of different strains of *C. pneumoniae* have indicated virtual identity among these strains (2, 3). We conclude, therefore, that the species *C. pneumoniae* cannot be amplified.

The presence or absence of cognate sequences in chlamydial DNA facilitates strain differentiation by PCR in its own right. Strain differentiation based solely on positive or negative amplification, however, requires cultivation of chlamydiae to yield quantifiable amounts of chlamydial DNA, as well as numerous controls. It is therefore preferable to use amplified DNA fragments for typing. Restriction enzyme analysis is one of the possible tools.

This investigation reveals a relationship between avian and mammalian serovar 1 chlamydial isolates. Despite the remarkably high conservation of MOMP gene sequences, distinct *Hae*III restriction patterns were generated from different serovars of *C. psittaci*. Avian and mammalian biovar 1 MOMP genes can be subdivided by *Hae*III restriction. The MOMP gene sequences of four strains of *C. psittaci* are known (8, 18, 29). The avian meningopneumonitis isolate (29) and strain A22/M from ovine abortion (18) can be amplified, according to the sequence data. They exhibit one identical *Hae*III restriction site, which generates fragments similar to those of 6BC and BM-Z1121. On the basis of the target sequence of primer 3GPB, another ovine abortion strain, *C. psittaci* isolate S26/3 (8), may be amplifiable as well. This MOMP gene differs from A22M, exhibits no *Hae*III restriction site, is identical in partial MOMP gene sequence to B-577, and thus may be related to B-577 and wt parakeet.

MOMP genes of both the Fe-Pn and GPIC serovars possess unique *Hae*III restriction patterns. Sequences of MOMP genes of various *C. psittaci* strains will reveal the divergence among VD, which determine the serovars, and conserved regions and whether this restriction fragment length polymorphism differentiation scheme conforms with antigenic differences. The present results indicate that PCR amplification, coupled with restriction analysis of the amplified MOMP gene, provides a simple and valuable tool for strain differentiation.

The external primers of this investigation were derived from nontranslated regions 5′ and 3′ to the MOMP gene

which are usually involved in directing transcription. Thus, differential amplification may reflect differences in MOMP expression determining diverse biological characteristics of chlamydial strains.

A disadvantage of PCR-based DNA detection is the need to confirm the authenticity of the amplified products either in a Southern assay using DNA probes or by direct DNA sequencing. Recently, Kaneko et al. (11) demonstrated that two-step PCR amplification with two successive sets of primer pairs and ethidium bromide staining of the amplification products is equivalent to one round of PCR and Southern blot hybridization analysis. This assay is equal in sensitivity and specificity to hybridization analysis and can be completed in 1 day. One internal primer combined with one of the external primers yielded a specific secondary fragment in our investigation. The amount of external primers transferred into the second PCR did not visibly influence the results. Obviously, the use of one new primer is sufficient to prevent preferential amplification of aberrant PCR products. A single, strongly predominant, gel-visualized DNA fragment of the expected size in secondary PCR yields information equivalent to that of Southern hybridization. Two regions corresponding to oligonucleotides precisely spaced on the same DNA molecule are required for specific exponential amplification. Southern blot hybridization using oligonucleotide probes is often ambiguous because of the high background at a low stringency of hybridization.

The results of this study confirm the heterogeneity of *C. psittaci* as demonstrated by DNA-DNA hybridization (3, 5). Representative strains of serovars with broad host and disease ranges could be amplified, while strains of possibly highly evolved serovars with narrow host or disease specificities were refractory to amplification. This PCR method is valuable despite its limitations. It is capable of detecting with high sensitivity strains of *C. psittaci* which have been associated with human disease and are most prevalent in infection of domestic animals. It will be necessary to develop PCR protocols with broadened specificity to trace chlamydial infections caused by all established serovars and, possibly, by as yet unidentified chlamydial strains in diverse hosts.

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