Identification of Individual Genotypes of *Chlamydia trachomatis* from Experimentally Mixed Serovars and Mixed Infections among Trachoma Patients

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Genetic polymorphisms in the major outer membrane protein gene (*omp-1*) of *Chlamydia trachomatis* B and Ba serovars have been demonstrated in Tunisian isolates. A total of 15 of 27 unique sequence signatures or *omp-1* genotypes were identified. However, differentiation of unique signatures from sequences that reflect those of strains involved in a mixed infection is necessary to define the molecular epidemiology of chlamydial ocular infections. We devised a strategy for identifying mixed infections by characterizing their effects on *omp-1* genotyping. Various ratios of elementary bodies from organisms of serovars A, B, Ba, and C that cause trachoma were amplified by PCR and were subjected to automated and manual sequencing. Serovar-specific primers were also designed so that each serovar could be individually amplified and its *omp-1* genotype unequivocally determined. One of 27 Tunisian samples showed a mixed infection with sequences comparable to those of serovars B and D. The *omp-1* genotypes of organisms involved in mixed infections can be accurately identified by automated sequencing and will be useful for molecular epidemiologic studies of populations worldwide who live where trachoma is endemic.

Research efforts have recently been directed toward an understanding of the molecular epidemiologic characteristics of *Chlamydia trachomatis* (7, 8). However, it has been difficult to precisely type and classify all serovars that are responsible for each type of chlamydial disease and those that may be site specific within the human body. This difficulty is due in part to an inability of standard serotyping assays which use serovar- or subgroup-specific monoclonal antibodies (MAbs) to consistently identify new serotypes or subtypes of existing serovars. Furthermore, in an increasing number of cases, a proportion of clinical isolates is found to be undefined variants (3, 7, 14). This has probably occurred because, although MAbs are directed toward the major outer membrane protein (MOMP), which is the most antigenically diverse surface protein of *C. trachomatis*, these MAbs have been developed only against the known prototype serovars of chlamydiae. Also, the MOMP-specific MAbs have not been standardized among different laboratories. This limits comparison and characterization of *C. trachomatis* isolates of the same or closely related serovars from different geographic regions. The new serovars or subtypes of *C. trachomatis* that have been identified were detected by the observation that some samples did not react to a certain MAb or reacted to a number of different MAbs in a unique way (2, 17, 19). Only through propagation of these strains, nucleotide sequence analysis, and development of new MAbs directed against surface antigens was it possible to classify these strains (6, 11, 18).

An additional complication of immunotyping is the potential for the lack of identification of infections caused by mixed serovars. Currently, MOMP-specific MAbs are used in a dot enzyme-linked immunosorbent assay or radioimmunoassay format to detect infections caused by multiple serovars. Yet, a

particular reaction pattern to different serovars is suggestive only of a mixed infection (2). These infections can, however, be confirmed by double-label staining of chlamydial inclusions by using fluorescein and rhodamine. Immunotyping requires propagation of the organism, in which only 42 to 80% of isolates reach a sufficient concentration for immunotyping (1, 14, 17). Furthermore, lower rates of isolation have been found for patients with previous histories of sexually transmitted diseases or chlamydial genital tract infections that have occurred within the 3 to 6 months preceding examination (10, 13). In vivo, patients with mixed infections are probably not infected with equal quantities of organisms of each serovar, and therefore, a few passages could result in the overgrowth of one serovar with the loss of recognition of a second serovar. This may be an even greater problem in identifying mixed infections in populations with trachoma in which certain serovars responsible for trachoma are known to propagate poorly in tissue culture. Indeed, a number of investigators have found a wide range of chlamydial recovery rates (37 to 76%) for patients with active inflammatory disease (4, 5, 12, 15). This may reflect a problem with clinical diagnosis, transport of specimens, or culture facilities in the field.

It is estimated that approximately 2 to 5% of chlamydial genital infections occur as mixed infections (1, 2). However, the same epidemiologic information is not available for populations with trachoma. We developed an alternative method for typing chlamydiae. The method involves amplification of the MOMP gene (*omp-1*) and sequencing by automation (*omp-1* genotyping) without the need for prior propagation of the organism in tissue culture (7). Using this technique, we identified unique sequence signatures among B and Ba serovars from trachoma patients that differ from the prototype serovar sequence by one to four amino acids. We report here the use of *omp-1* genotyping to reliably differentiate the sequences of organisms involved in mixed infections from novel sequence signatures or new *omp-1* genotypes and to

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FIG. 1. Schematic of the four VSs of the MOMP gene (*omp-1*) and the locations of the 5' and 3' oligonucleotide primers. MF21 is a universal 5' upstream primer which is used in all PCRs except for amplifications of VS3 and VS4, in which MVF3 was used as the 5' upstream primer. Primers are denoted by a line with an arrow at the beginning (3') or end (5') of the line. MF21 and MB22 amplify VS1 and VS2, respectively; MVF3 and MB4 amplify VS3 and VS4, respectively; MF21 and A1 amplify only serovar A; MF21 and B1 amplify only serovars B and Ba; MF21 and C1 amplify only serovar C; and MF21 and D1 amplify only serovar D.

detect mixed serovars in specimens from a population in Tunisia where trachoma is endemic.

MATERIALS AND METHODS

***C. trachomatis* strains.** The *C. trachomatis* strains used in the study were A/HAR-13, B/TW-5/OT, Ba/AP-2, and C/TW-3/OT as well as 16 ocular specimens from Tunisia (8116, 8128, 8137, 8138, 8145, 8146, 8147, 8148, 8149, 8166, 8173, 8179, 8180, 8181, 8182, 8193) collected during 1975 as described previously (7). The samples from Tunisia were immunotyped as B and Ba serovars (7).

Preparation of samples containing mixtures of serovars. Elementary bodies (EBs) from strains of serovars A (A/HAR-13), B (B/TW-5/OT), Ba (Ba/AP-2), and C (C/TW-3/OT) were quantitated by first diluting 1 μ l of unpurified seed EBs in 99, 499, and 999 μ l of phosphate-buffered saline (PBS). Four microliters of each dilution was placed within an etched circle on a glass slide, air dried, and fixed with methanol. Fluorescein isothiocyanate antibody (Microtrak Kit; Syva, Palo Alto, Calif.) to chlamydial MOMP was added to cover the etched circle, and the slides were incubated at 37°C for 30 min. The slides were washed with PBS, air dried, and read with a fluorescent microscope by using a $\times 100$ oil immersion lens. All fluorescing EBs within the circle were counted to quantitate the number of EBs per microliter of each serovar stock. Equal quantities of two different trachoma serovars were then combined in a final volume of 100 μ l of PBS to stimulate a mixed infection (A and B, A and Ba, A and C, Ba and B, Ba and C, and B and C). Unequal ratios of EBs from these serovars were also combined (1/10, 1/20, 1/30, 1/50, and 1/100) for subsequent testing.

PCR protocol. Two universal sets of oligonucleotide primers flanking variable segments (VSs) 1 and 2 (MF21 and MB22) and VSs 3 and 4 (MVF3 and MB4) were used for amplification as described previously (7) (Fig. 1). These primers uniformly amplify all prototype serovars (A to K and L1 to L3, including Ba, Da, Ia, and L2a) of *C. trachomatis* without differential

amplification of VSs by serovar (unpublished data). In addition, four serovar-specific primers for serovars A (VS2), B and Ba (VS3), C (VS4), and D (VS4) were designed on the basis of the unique sequences of each serovar in the respective VS designated in parentheses (Table 1; Fig. 1). The nucleotide positions of all primers except for A1 and D1 were based on the sequence described by Stephens et al. (16). Primers A1 and D1 were based on the sequence described by Yuan et al. (20). Primer MF21 was used as the 5' upstream primer in each of the serovar-specific amplifications. All samples were subjected to PCR by using the first two sets of primer pairs (7) in two separate amplification reactions. Subsequently, the experimentally mixed samples, one Tunisian sample exhibiting a mixed infection (on the basis of manual reading of the automated sequence data), and the remaining Tunisian samples were amplified by using the four serovar-specific primer pairs. Clinical archival samples were prepared for PCR as described previously (7). Samples infected with mixed serovars were prepared by combining equal or unequal volumes of EBs, as described above, in a solution of equal volumes of TE (Tris Cl, [pH 8.3], 1 mM EDTA) and 40 mM dithiothreitol. These samples were then boiled for 10 min. One microliter of each sample or mixture was used in the PCR in a final reaction volume of a 100- μ l solution containing 50 mM KCl, 10 mM Tris Cl (pH 8.1), 2.5 mM $MgCl_2$, 100 μ M (each) dATP, dCTP, dGTP, and dTTP, 150 ng of each primer, and 2.5 U of *ampli-Taq* DNA polymerase (Perkin-Elmer Cetus, Emeryville, Calif.). Each reaction mixture was incubated at 95°C for 1 min prior to 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min; this was followed by incubation for 10 min at 72°C at the end of the last cycle. Ten microliters of each reaction product was run on a 1.5 or 2% agarose gel to confirm the size of the PCR product.

Manual and automated sequencing. The amplification products of each PCR were purified by using the GeneClean (GeneClean; Bio 101, La Jolla, Calif.) protocol in the package insert. Approximately 60 ng of each template was then se-

TABLE 1. Oligonucleotide primers for the amplification of *omp-1*

Primer (nucleotide position)	Sequence (5'–3')
MF21 ^a (209–233)	5'-TGTAAAACGACGGCCAGTGCCTGACCGCGCTCTTGAAAACAGATGT
MVF3 ^b (535–555)	5'-TGTAAAACGACGGCCAGTGCCTGACCGCGCTCTTGTTGGGAATGT
A1 ^c (537–516)	5'-CAAAGCAGTGTTAGGAACAATA
B1 (759–735)	5'-GTAAGATCAAGAGGCAATTCTCTTA
C1 (1021–1000)	5'-TCAGCCAGTTCGTTATCGGTTC
D1 (955–933)	5'-GGTAGTATCAAAAATAGCTGTA

^a MF21 is the 5' upstream primer which amplifies all known serovars; the underlined section is the sequence of the M13 universal primer.

^b MVF3 is the 5' upstream primer which amplifies VS3 and VS4 for all known serovars; the underlined section is the sequence of M13 universal primer.

^c A1, B1, C1, and D1 are the 3' downstream primers which amplify only the respective serovar.

quenced manually and by an automated means as described previously (7). Briefly, the templates were annealed to M13 universal primers, amplified by PCR, and loaded onto the appropriate gel for manual (6) and automated sequencing. The results obtained by automated sequencing were read both by the computer and by eye in a blinded fashion. We have recently improved the automated sequencing assay to incorporate dye-terminal labeling according to the manufacturer's instructions (model 373A DNA Sequencing Manual; Applied Biosystems, Foster City, Calif.), which has facilitated the processing of multiple samples, including the sequence identification of each serovar in a mixed infection.

RESULTS

Sequencing patterns of experimentally mixed EBs. The VSs of *omp-1* were amplified by using the two sets of *omp-1* primers that would amplify DNAs from all serovars for each pair of serovars that had been mixed in equal or unequal ratios. Automated sequencing results showed the predicted number and location of the expected dual peaks in single nucleotide lanes only when there was a difference in sequence between the two serovars. The automated sequencing results for unequal ratios of mixed serovars (i.e., 1/10, 1/20, 1/30, and 1/50) showed dual peaks up to and including the 1/30 or 30/1 ratios of mixed EBs. Thereafter, the most abundant serovar was solely represented in the sequence. For manual sequencing, equal ratios of both serovars revealed two bands at a single nucleotide position when the serovar sequences differed. In some cases, four bands appeared, and these bands were similar to stops at GC-rich regions, which has been observed occasionally when a single serovar is sequenced. When unequal ratios of serovars were sequenced manually, a single sequence appeared at the 1/10 or 10/1 ratios, with excellent band resolution and no stops.

***omp-1* genotyping of samples from Tunisia.** Twelve samples from Tunisia were subjected to *omp-1* genotyping by using the two sets of *omp-1* primers that would amplify DNAs from all serovars as described above, and the genotypes were compared with those of 15 other samples from Tunisia whose genotypes were determined previously with the same set of primers (7). In 1 of the 27 samples, two peaks were detected at single nucleotide positions by automated sequencing. This finding suggested the presence of more than one genotype in the sample (i.e., a mixed infection). To obtain discrete, unequivocal sequences, this sample was then subjected to amplification by using the three serovar-specific primers for serovars A, B and Ba, and C, with primer MF21 used as the 5' universal upstream primer in one amplification reaction. A previous experiment had been performed with these three sets of primers on experimental mixtures of serovars A, B, and C in one PCR (Fig. 2). Each primer pair amplified a segment of *omp-1* of a different size, whereby three distinct bands were easily distinguished on a 1% agarose gel (Fig. 2). The lane labeled P in Fig. 2 represents this reaction on the gel. Each serovar-specific primer was also used in PCR to amplify, for example, serovar B if the primer pair was not specific for serovar A or C (Fig. 2). These reactions are labeled 1 through 6 in Fig. 2. They represent primer A1 with primer MF21 in a PCR with DNAs from serovars B and C in lanes 1 and 2, respectively; primer B1 with primer MF21 in a PCR with DNAs from serovars A and C in lanes 3 and 4, respectively; and primer C1 with primer MF21 in a PCR with DNAs from serovars A and B in lanes 5 and 6, respectively. In each combination, the serovar-specific primer failed to amplify the DNA from the heterologous serovar. This showed that the

FIG. 2. One percent agarose gel electrophoresis analysis of PCR products by using 3' downstream serovar-specific primers with the *C. trachomatis* 5' upstream universal primer MF21 and DNAs from serovars A, B, and C. Lanes are labeled as follows: MW, molecular weight markers consisting of a *Hae*III digest of pBR322 DNA and a *Hind*III digest of bacteriophage λ DNA; P, PCR product resulting from amplification of an equal mixture of lysed EBs from serovars A, B, and C by using primers MF21, A1, B1, and C1 (Table 1) together in one reaction; A, PCR product resulting from amplification of DNA from serovar A by using primers MF21 and A1; B, PCR product resulting from amplification of DNA from serovar B by using primers MF21 and B1; C, PCR product resulting from amplification of DNA from serovar C by using primers MF21 and C1; 1 and 2, PCR of DNAs from serovars B and C, respectively, amplified by using primers MF21 and A1; 3 and 4, PCR of DNAs from serovars A and C, respectively, amplified by using primers MF21 and B1; 5 and 6, PCR of DNAs from serovars A and B, respectively, amplified by using primers MF21 and C1.

serovar-specific primers were specific for the respective serovars. For the Tunisian sample, only the serovar B and Ba primer set amplified the DNA, which subsequently revealed a sequence identical to the published sequence of serovar B (16). In analyzing the mixed sequence of this sample from the first two sets of primer amplifications, the sequence appeared to be present along with a sequence identical to the published sequence of serovar D (20) at nucleotide positions where there were two peaks (Fig. 3). These dual peaks occurred at predicted positions where there was a difference in sequence between the two serovars (Fig. 3). Therefore, a serovar D-specific downstream primer was synthesized (Table 1) and used to amplify the sample. In this case, the results indeed showed an unambiguous sequence which was identical to that of serovar D (20).

To examine the specificity of the serovar-specific primers, all 26 Tunisian samples (none showing evidence of mixed infection by automated sequencing) were amplified with the four serovar-specific primers. Only the serovar B and Ba primer pair produced a PCR product. The sequence of each product was found to contain a sequence with a single genotype, which is consistent with previous sequencing results (7). These results confirmed the specificity of the serovar-specific primers.

DISCUSSION

Determination of the genetic and immunologic relatedness of chlamydial serotypes has become important as new serovars and subtypes of known serovars are identified as pathogens in

FIG. 3. Section of automated sequence from VS2 of an archival Tunisian sample which exhibited a mixed serovar infection. Numbers at the top corners of the box denote the nucleotide positions in VS2. Question marks denote the positions of dual peaks at nucleotide positions 495, 497, 498, and 501, where the sequences of serovars B and D differ (20). Dual peaks can also be discerned at nucleotide positions 489, 492, and 500 by semiautomation or visual inspection of the sequence; these occur at predicted positions where the two sequences differ (20). The color for each nucleotide is represented at the top of the sequence. Note that at position 495, red (T) is superimposed on yellow (G), which makes the latter more difficult to see.

human disease. Efforts at typing chlamydiae by using the phenotypic characteristics of the organism, such as the serovar MOMP antigens, are important but are limited by those MAbs that are available for immunotyping, their spectra of reactivities to the known MOMP antigens, and the need for propagation in tissue culture. Recently, analysis of *omp-1* DNA has become an important alternative to immunotyping. Advances in DNA technology, which include automated sequencing, have made this technique a feasible and relatively routine means of generating gene sequences for typing organisms in a short period of time. We have exploited this technology to develop an *omp-1* genotyping assay for chlamydiae (7) and used that assay in the present study to detect experimentally mixed serovars and a mixed infection from a trachoma patient in Tunisia. This approach defines a new direction for epidemiologic and clinical microbiologic studies of trachoma throughout the world.

When screening clinical samples for the presence of geno-

types, it will be important to differentiate mixed from non-mixed infections. This will provide a clearer picture of the epidemiology and natural history of chlamydial diseases. Furthermore, the ability to detect strains that are present at a 30-fold lower level than the predominant genotype will impart a greater level of confidence in the automated sequencing results that are obtained, since *in vivo*, mixed infections most likely do not contain equal concentrations of serovars. However, it is possible that other variants could be present at even lower concentrations in clinical samples and that they could be detected by using serovar-specific primers for all known serovars. Yet, the importance of the presence of a lower concentration of one serovar in a mixed infection may be difficult to ascertain. Lower concentrations may represent a persistent or latent yet infected state which is maintained in the host until certain stimuli induce growth and replication. In a recent study by Holland et al. (9) involving experimental ocular infections in cynomolgus monkeys, chlamydial DNA and RNA were detected up to 5 weeks after cultures of samples from monkeys who exhibited either clinically active or inactive disease became negative. However, these animals were not followed for the possible recurrence of infection or disease (if this occurs *in vivo*) beyond the time when all laboratory tests became negative. Although the investigators' data (9) suggest that the organism can persist in host tissues at least for a short period of time, it is unclear whether the inability to culture the organism from a clinical sample is merely an issue of the presence of insufficient numbers of organisms for cell culture isolation and/or the presence of host-induced inhibitors that are carried over into culture or whether these chlamydiae are noninfectious or nonviable. *omp-1* genotyping may be an appropriate approach to evaluating the question of persistence or latency of infection. For example, in a population that is known to contain different chlamydial genotypes, a prospective epidemiologic study of individuals whose infections are revealed by the presence of organisms with identical *omp-1* genotypes pre- and posttreatment (treatment failures) would support the concept of latency or persistence. In any case, the viabilities of low concentrations of organisms, if they exist, and their importance in infection and disease will need to be addressed in future studies.

Fifteen archival trachoma strains from Tunisia were previously genotyped and found to contain unique sequence signatures in comparison with the known sequences of serovars B and Ba. The organisms in an additional 12 specimens from Tunisia were sequenced. Fifteen of these 27 specimens were found to contain organisms with unique *omp-1* genotypes. In addition, 1 of the 27 samples showed evidence of a mixed infection by inspection of automated sequencing data for the infecting organism. Using serovar-specific primers and automated sequencing, we were able to determine that this sample did indeed contain organisms with two different sequences identical to the sequences of serovars B (16) and D (20). The discovery of a sequence identical to that of serovar D was unexpected. However, serovars D and Da have previously been identified in areas where trachoma is endemic (18). This emphasizes the need for evaluating specimens not only for trachoma serovars but also for *C. trachomatis* organisms which are transmitted sexually. Furthermore, although the sample size for trachoma specimens in the present study was small, the prevalence of mixed ocular infections was apparently low (i.e., 3.7%) and was similar to the 2 to 5% rate observed for mixed chlamydial infections in populations of patients with sexually transmitted diseases (1, 2). However, the rates of chlamydial infections in populations with sexually transmitted diseases may be artificially low since they were based on immunotyping

of propagated samples. In regions where trachoma is endemic, it has been thought that one or two serovars predominate in any region. Yet, we found multiple, albeit similar, genotypes of serovars B and Ba in archival trachoma isolates (7). Furthermore, more than 90% of these nucleotide sequence changes encoded for amino acid substitutions. Some of the newer subtypes of existing serovars that have recently been identified serologically have also been found to contain single amino acid changes in MOMP. Serovars D and Da and L2 and L2a are examples of this (6, 11). Thus, the finding of only a few or even one amino acid change in the MOMP sequence of an isolate may translate into significant immunologic differences.

The identification of new genotypes of serovars B and Ba in specimens from Tunisia and a serovar D isolate in the present study, which was present as a mixed ocular infection, suggests that there is a larger array of antigenic types than was previously recognized in populations with trachoma. It will be especially important to evaluate the infections caused by those organisms that are presumed to be transmitted primarily sexually but which may actually cause serious ocular disease. Additional studies are required to define the prevalence and clinical significance of mixed ocular infections, the characteristics of the genotypes involved, and the importance of such infections in the transmission of trachoma in the respective households and communities where trachoma is endemic. Such molecular studies should provide a more comprehensive understanding of the epidemiology of trachoma than can be achieved by antibody typing approaches.

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