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General colorimetric method for DNA diagnostics allowing direct solid-phase genomic sequencing of the positive samples

(*Chlamydia trachomatis*/biotin/polymerase chain reaction/Lac repressor–enzyme conjugate)

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ABSTRACT **A system for rapid colorimetric detection of specific genome DNA fragments amplified by the polymerase chain reaction (PCR) is described that has been designed to allow direct solid-phase sequencing of positive samples. The amplified material is immobilized on magnetic beads by using the biotin streptavidin system. An *Escherichia coli lac* operator DNA sequence is incorporated in the amplified material during the second step of a nested primer procedure. This 21-base-pair sequence is used for a general colorimetric detection with a fusion protein consisting of the *E. coli* Lac repressor and β-galactosidase. Positive samples can be treated subsequently with alkali to obtain a single-stranded DNA template suitable for direct genomic sequencing. This method to detect immobilized amplified nucleic acids (DIANA) is well adapted for automated or semiautomated clinical assays. Here, we show that it can be used to detect and sequence *Chlamydia trachomatis* genomic DNA in clinical samples.**

Analysis of specific nucleotide sequences using the polymerase chain reaction (PCR) has many potential applications in mass screening of various pathogens, genetic disorders, and allelic variations (1). For detection of pathogens, this *in vitro* amplification technique has many advantages compared with conventional methods because of the generality and sensitivity of the assay. In addition, the PCR procedure potentially allows direct genomic sequencing of the amplified material. This makes it possible to perform epidemiological studies in which polymorphic regions of the pathogen genome are sequenced and analyzed. In addition, studies of bacterial and viral responses to drugs are simplified. Thus, it is possible to determine the sequence of genomic regions possibly responsible for drug resistance of pathogens isolated from patients.

However, most procedures for detection of products of PCR are not well suited for mass screening as they involve electrophoresis, radioactive isotopes, or centrifugations (2). Even though genomic sequencing of positive samples already has been incorporated into an assay (3), a system for colorimetric detection and sequencing of *in vitro* amplified DNA sequences that is specific, rapid, and suitable for automation would be advantageous. Here, we describe a solid-phase approach to obtain such a system, designated "detection of immobilized amplified nucleic acids" (DIANA). The assay is exemplified by the detection and sequencing of genomic DNA from *Chlamydia trachomatis* in clinical urogenital samples. This analysis was chosen as a model system because a rapid assay for diagnosis of *C. trachomatis* is clearly needed. Current methods are based on isolation of pathogens through cell culture, a technique that is both cumbersome and time consuming (4).

MATERIALS AND METHODS

Bacterial Strains. A strain of *C. trachomatis* biovar L2 was kindly supplied by H. Gnarpe (Gävle Hospital, Sweden). Clinical samples were obtained with cotton-tipped swabs from male urethra (Karolinska Hospital, Stockholm, Sweden) and stored in PCR buffer (see below) at 4°C.

Purification of the Fusion Protein. *Escherichia coli* XA90/Δ14 (5) containing the *lacI* and *lacZ* gene fusion encodes a fusion protein with both LacI repressor and β-galactosidase activity. The *E. coli* strain was grown overnight in baffled Erlenmeyer flasks containing tryptic soy broth (30 g/liter; Difco) containing yeast extract (7 g/liter; Difco) at 37°C. Cell extracts were obtained by sonication (6) in TST buffer (0.1 M Tris·HCl, pH 7.5/0.15 M NaCl/0.1% Tween) containing 0.1% bovine serum albumin, 0.1 mM $ZnCl_2$, 1.0 mM $MgCl_2$, and 10 mM 2-mercaptoethanol. The fusion protein was purified by $(NH_4)_2SO_4$ precipitation, and the fraction between 25% and 45% $(NH_4)_2SO_4$ saturation was used, followed by affinity chromatography on a heparin-Sepharose column (Pharmacia). The fusion protein was eluted in 0.25 M KCl/15% (vol/vol) glycerol buffer.

Synthesis of Oligonucleotides. Four oligonucleotides (RIT23–RIT26) complementary to *C. trachomatis* genomic DNA (7) and one sequencing primer (RIT43) were synthesized by phosphoramidite chemistry on an automated DNA synthesis machine (Gene Assembler Plus, Pharmacia) as described by the manufacturer. Two oligonucleotides (RIT25 and RIT43) were synthesized with amino groups in the 5′ end, which was used to introduce a biotin derivative, Biotin-X-NHS ester (Clontech), and a fluorescent label, fluorescein isothiocyanate (FITC; Pharmacia), respectively, as described by the manufacturers. RIT25 and RIT43 were purified by using a fast protein liquid chromatography (FPLC) pepRPC 5/5 column (Pharmacia).

PCR Amplification. The *in vitro* amplification was performed with a Techne Programmable Dri-Block PHC-2 (Techne, U.K.). The amplification was run in two consecutive steps; first, 35 cycles with RIT23 and RIT24 and, after a 1:100 dilution, a second amplification for 15 cycles with RIT25 and RIT26. The PCR buffer used contained 20 mM 3-{[tris(hydroxymethyl)methyl]amino}-1-propanesulfonic acid (Taps) (pH 9.3), 8 mM $MgCl_2$, 25 mM KCl, and 0.2% Triton X-100. The clinical swabs were put into PCR tubes filled with PCR buffer (10 μl) and rubbed against the wall of the tube. The tube was subsequently heated to 99°C for 5 min to lyse the cells and then cooled to 0°C. To the lysed mixture, 90 μl of a PCR buffer with 0.2 mM dNTP, 0.2 mM of each primer, and 1.0 unit of *thermus aquaticus* AmpliTaq polymerase (Perkin–Elmer) were added. The reaction mixture was covered with a layer of light mineral oil (Sigma). One temperature cycle on the PCR was: denaturation of template

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Abbreviations: PCR, polymerase chain reaction; DIANA, detection of immobilized amplified nucleic acids; CrP, cysteine-rich protein.
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at 95°C for 0.5 min; annealing of primers at 58°C for 1 min; and extension of primers at 72°C for 1 min. The size of the PCR product was analyzed by 1% agarose gel electrophoresis.

Immobilization on Magnetic Beads. Magnetic beads with covalently coupled streptavidin, Dynabeads M280-streptavidin (8), were obtained from Dynal AS (Norway). A neodymium–iron–boron permanent magnet (Dynal AS) was used to sediment the beads during the washing procedures. Beads (300 μg) were mixed with 85 μl of the PCR mixture and incubated for 20 min at room temperature. The beads were washed twice with 0.1 ml of TST buffer.

Enzymatic Detection. The beads with the immobilized DNA were mixed with 200 μl of fusion protein (LacI-LacZ, 0.2 mg/ml) and 200 μg of sonicated herring sperm DNA in a Eppendorf tube for 30 min. The beads were washed four times with 0.5 ml of TST buffer. The substrate, *o*-nitrophenyl β-D-galactoside, was added, and the change of absorbance at room temperature was measured. One unit is defined as the change of one absorbance unit per minute at 405 nm.

Solid-Phase DNA Sequencing. The positive samples were sequenced by melting the two strands of the immobilized DNA with 50 μl of 0.15 M NaOH at room temperature in 10 min. Thereafter the beads were washed once with 50 μl of 0.15 M NaOH and three times with 50 μl of TE buffer (0.1 M Tris·HCl, pH 7.5/1 mM EDTA). The annealing of the fluorescent primer was performed in a buffer containing 10 mM Tris·HCl (pH 7.5), 100 mM NaCl, 10 mM $MgCl_2$, 0.1 mg of bovine serum albumin per ml, and 2 pmol of RIT43 in a total volume of 17 μl. The annealing mixture was heated to 65°C and allowed to cool to room temperature. Then, 1 μl of MID [manganese (II), isocitric acid, and dithiothreitol] solution (Pharmacia) and 3 units of T7 DNA polymerase (Pharmacia) were added to the annealing mixture to a total volume of 20 μl, of which 4.5 μl was mixed with 2.5 μl of sequencing mixtures (A, C, G, and T) from Pharmacia's Autoread T7Sequencing kit (Pharmacia) and incubated 10 min at 37°C. Thereafter the beads were heated to 85°C with 5 μl of deionized formamide to denature the double strands. After sedimentation, 4 μl of the supernatant was loaded on a 6% polyacrylamide gel on the automated laser fluorescence (ALF) sequencing apparatus (Pharmacia LKB).

RESULTS

The Basic Concept. Our approach for the detection and sequencing of specific *in vitro* amplified material using a solid phase is shown in Fig. 1 (see steps *i–vii*). The first step (*i*) is a standard PCR with oligonucleotides specific for the target DNA sequence. A large number of cycles (i.e., 25–35) are performed to obtain many template molecules that can be used in the second PCR step. For samples lacking the target DNA, a nonspecific amplification of random DNA will occur as described earlier (3). The material obtained after the first PCR step is diluted and subsequently used in a second PCR step (*ii*) with the inner primers, which anneal to sequences within the DNA fragment amplified in the first step. One of the primers is biotinylated, while the other contains a *lac* operator "handle" consisting of 21 nucleotides. Thus, successful amplification yields specific DNA with biotin and *lac* operator incorporated in the fragment. The second PCR reaction is carried out with less cycles (i.e., 15); therefore, a very low yield of fragments containing biotin and *lac* operator will be obtained for samples containing nonspecific DNA.

The biotinylated material is subsequently captured on magnetic beads (*iii*) coupled with streptavidin by using the specific interaction between biotin and streptavidin. Thus, nonbiotinylated fragments can easily be removed. A recombinant fusion protein consisting of the lac repressor (LacI) and β-galactosidase (LacZ) is added to the beads (*iv*), whereafter the bound enzyme conjugate is detected by adding a chromogenic substrate (*v*) specific for the β-galactosidase enzyme.

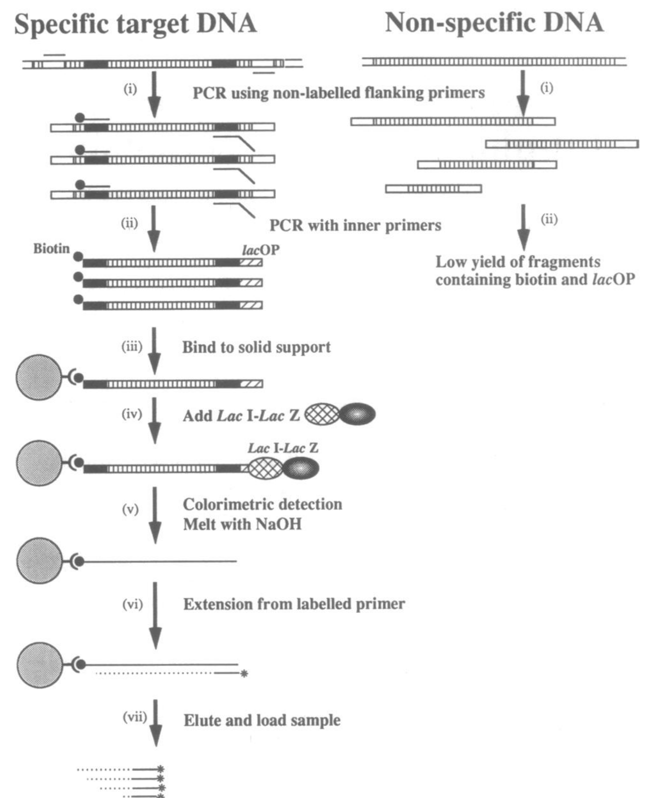

FIG. 1. Schematic drawing outlining the concept for fluorescent DIANA followed by direct solid-phase sequencing. The boxes represent DNA sequences—white for outer primers, black for inner primers, and striped for the *lac* operator. See the text for details.

The samples identified as positive by this colorimetric procedure can be treated directly with alkali to remove the bound fusion protein and to melt the double-stranded DNA immobilized to the beads (*v*). The biotin–streptavidin complex is resistant to this treatment; thus, a single-stranded template suitable for sequencing is obtained by using this one-step elution procedure. Therefore, a solid-phase DNA sequencing protocol (9) can be followed without the need for cloning procedures or extra template preparations. The extended material (*vi*) is eluted from the beads with formamide and loaded on a sequencing gel (*vii*). The solid-phase method provides a pure template (9), which ensures that reproducible sequence information is obtained without the need for precipitations, centrifugations, or desalting procedures.

Design of the Synthetic Primers. *C. trachomatis* is a Gram-negative bacterium characterized by an obligatory parasite life cycle within eucaryotic host cells. On the basis of clinical, biological and molecular characteristics, the human *C. trachomatis* isolates have been grouped in two varieties determined biologically (biovars) and 15 varieties determined serologically (serovars) (10, 11). Serovars L1, L2, and L3 are clinically important as they are associated with a relatively invasive form of chlamydia disease involving lymphoid tissue (12).

Several genes from serovars L1 and L2 of *C. trachomatis* have been isolated and characterized. This includes the major outer membrane protein from serovar L1 (13) and L2 (14) and the outer membrane cysteine-rich protein (CrP) from serovar L1 (7). We decided to perform a DIANA assay as described in figure 1 with *C. trachomatis* as a model system with the gene for CrP as the target DNA. CrP has been suggested to be necessary for the structural integrity of the chlamydial elementary body envelope (15), which makes the protein particularly important, given the apparent absence of peptidoglycan in *C. trachomatis* (16). Therefore, the gene encod-

ing CrP is most likely essential for invasive chlamydia and is a potential candidate as a target for detection of chlamydial DNA in clinical samples.

Four oligonucleotide primers specific for the middle part of the CrP gene coding region of *C. trachomatis* serovar L1 were synthesized. The sequences of the four primers are shown in Fig. 2, where also the location of the target DNA within the CrP gene is shown. The two outer primers hybridize 276 base pairs from each other, while the inner primers are 243 base pairs apart. A biotin derivative was covalently coupled to one (RIT25) of the inner primers, whereas a 21-base-pair handle corresponding to the *E. coli lac* operator sequence was added to the 5′ end of the other inner primer (RIT26).

Immobilization of the PCR-Amplified Material on Magnetic Beads. To test the efficiency of the primers designed to amplify the *Chlamydia* CrP gene coding region, a culture of *C. trachomatis* serovar L2 was used for a standard PCR agarose assay (1). Cells were directly lysed in the PCR buffer, and the CrP gene was amplified for 35 cycles with the outer primers (RIT23 and RIT24, Fig. 2). After a 1:100 dilution of the obtained material, a second PCR step was performed for 15 cycles with the inner primers (RIT25 and RIT26, Fig. 2). The material after the first PCR step (and also after the second PCR step) was mixed with magnetic beads containing streptavidin, and the supernatant was analyzed by agarose gel electrophoresis. The results (Fig. 3) show that the first PCR step yields a specific band (lane 3) of the expected size (318 base pairs), which does not bind to the magnetic beads (lane 4). In contrast, the shorter-sized (264 base pairs) material obtained after the second PCR step (lane 1) is efficiently captured by streptavidin-coated magnetic beads (lane 2). More than 95% immobilization yield is achieved, which demonstrates the efficiency of the biotin–streptavidin system for capturing DNA on a solid support.

Colorimetric DIANA of *Chlamydia* in Clinical Samples. Several clinical samples of human urogenital origin were used to investigate the DIANA concept as outlined in Fig. 1. A

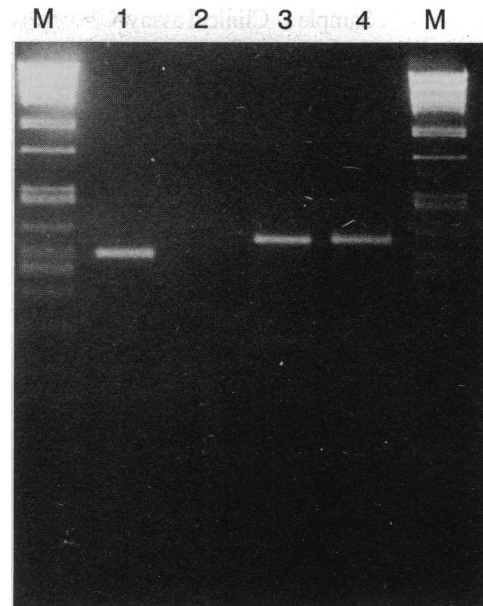

FIG. 3. A 2% agarose gel showing the binding of the amplified fragment to the beads and the size of the inner and outer fragment. Lanes: 1 and 2, material from the second amplification; 3 and 4, outer fragment. Lanes 1 and 3 correspond to unbound material, while lanes 2 and 4 correspond to the supernatant after binding to the beads has occurred. The marker is phage λ DNA digested with *Pst* I. Note that the length of the fragments is the expected size (264 and 318 base pairs, respectively).

standard cell culture assay was carried out in parallel on the same samples. The *C. trachomatis* serovar L2 cell culture (Fig. 3) was used as a positive control in the assay.

The nine samples were amplified as described, and the obtained material was immobilized to streptavidin-conju-

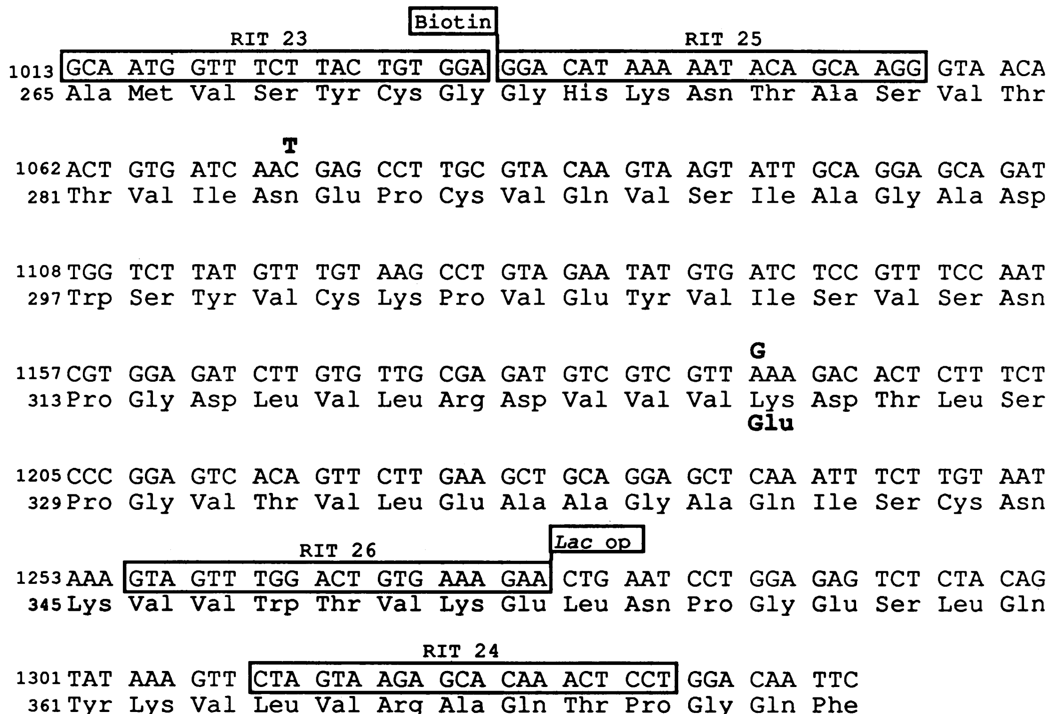

FIG. 2. Sequence of the CrP target gene of *C. trachomatis* and the primers used for detection (RIT23-26) and sequencing (RIT43). Mutations determined by genomic sequencing of the clinical samples (Fig. 4) are indicated. The numbers refer to the nucleotides as described by Clark *et al.* (7). Note that the sequences of primers RIT24 and RIT26 are complimentary to the sequence shown in the box. The *lac* operator sequence is TTAACACTCGCCTATTGTTAA-5′. The sequencing primer RIT43 (5′-(FITC-A)ATTGTTATCCGCTCACAATT-3′, where FITC is fluorescein isothiocyanate) hybridized with the *lac* operator, which is introduced in the second amplification.

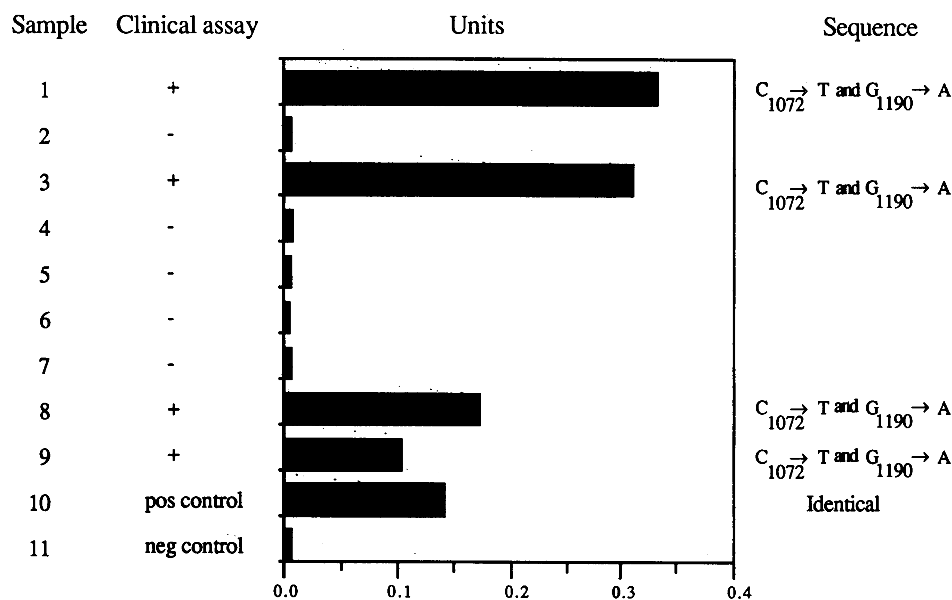

FIG. 4. Results of the DIANA assay on clinical *Chlamydia* samples. The results of the clinical assay performed by a standard cell culture technique are indicated (+/−). The activity of the DIANA is defined as described in *Materials and Methods*. Sample 10 is a positive control of cultivated *C. trachomatis*, and sample 11 is a negative control for the PCR reactions (no DNA template was added to this tube).

gated magnetic beads. The recombinant fusion protein consisting of the Lac repressor–enzyme conjugate was added. After a wash, a chromogenic substrate was added, and the change of absorbance (clearly visible) after a 1-min incubation at room temperature was detected. The results (Fig. 4) demonstrate a complete correlation between the DIANA assay and the conventional cell culture assay. The absorbances for the negative samples were very low and confirmed the low background levels obtained after a two-step PCR. Note that the positive samples varied in the level of the signal. These variations most likely reflect variations in the later nonexponential stages of the second PCR and are not due to differences in the amount of target DNA in the starting material.

Solid-Phase DNA Sequencing of Positive Samples. The positive samples obtained by the DIANA assay (Fig. 4) were all directly sequenced by the solid-phase procedure outlined in Fig. 1. One strand of the bound DNA fragment and the enzyme conjugate were eluted simultaneously with alkali, and the remaining immobilized strand was used as template for genomic sequencing. A fluorescent extension primer corresponding to the *lac* operator sequence was used. The separation of the extended material was carried out on an automated sequencing apparatus (Pharmacia LKB). A clear sequence was obtained for all positive samples, and the results for one of the samples (no. 1 in Fig. 4) are shown in Fig. 5.

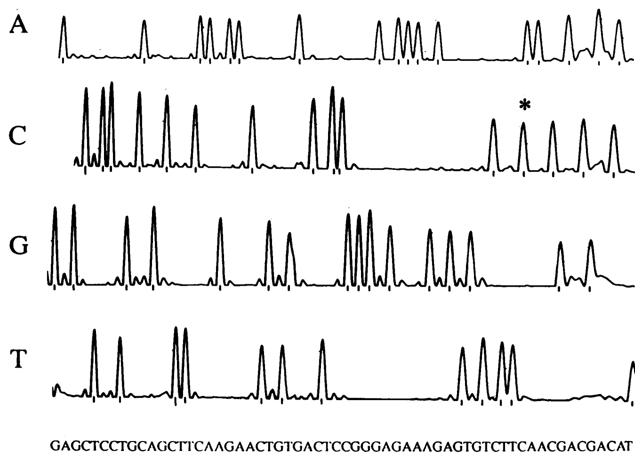

FIG. 5. The DNA sequences for one (no. 1 in Fig. 4) of the positive *Chlamydia* samples. The sequence was determined as described in Fig. 1 with a general RIT43 fluorescence-labeled primer. The analysis was performed on an automated laser fluorescence (ALF) sequenator (Pharmacia LKB).

Direct genomic sequencing revealed that the *C. trachomatis* serovar L2 control has a sequence that is identical to the one published by Clark *et al.* (Fig. 2, ref. 7). In contrast, all four positive samples (Fig. 4, samples 1, 3, 8, and 9) obtained from patients in Sweden have two mutations (Fig. 4) corresponding to Cyt-1072 → thymidine and Gua-1190 → adenosine. One of these point mutations (position 1072) is silent and does not alter the corresponding protein. The other mutation (position 1190) creates a change in the CrP from a basic lysine residue at amino acid position 324 to an acidic glutamic acid residue (Fig. 2). This point mutation is indicated by a star in Fig. 5. The question arises whether this change reflects a genetic difference that has immunological or clinical relevance.

DISCUSSION

The results presented in this paper show that a solid-phase approach can be used to combine a single colorimetric DIANA with a method for efficient direct genomic sequencing of positive samples. The assay combines the advantages of the PCR method with the high specificity and stability of the biotin–streptavidin system and the simplicity of using a colorimetric detection based on the β-galactosidase enzyme. The high binding constants for the biotin–streptavidin complex ($K_d = 10^{-15}\ M^{-1}$) and the *lac* repressor–operator complex ($K_d = 10^{-12}\ M^{-1}$) accentuate the specificity and the efficiency of the system. The magnetic beads as solid support ensures that no centrifugations, filtrations, or precipitations are needed. The DIANA assay can be performed in a few hours, and the complete assay, including sequencing of the positive samples, can be performed within a working day. Note that sequencing is performed directly on the amplified genomic DNA without the need for cloning procedures. Thus, the problems due to *Taq* polymerase misincorporation often encountered for PCR cloned material (17) are avoided.

We have shown with the described strategy that *Chlamydia* can be detected in clinical samples. Obviously, the same

approach can be used for detection of other viral, bacterial, protozoan, and fungal pathogens. Recently, similar approaches have been described for detection of malaria (18) and human immunodeficiency virus (19) by *in vitro* amplification followed by solid-phase capturing and colorimetric detection. However, both of these assays involved a capturing system based on DNA binding proteins; thus, it was not possible to continue with direct genomic sequencing. In addition, the solid support used here was monodispersed magnetic beads with covalently linked streptavidin, which is more convenient than the Sepharose beads used by Lundeberg *et al.* (18) and gives higher binding capacity (J.W. and J.L., unpublished data) than does coating the walls of microtiter wells as described by Kemp *et al.* (19). This is important, as a high binding capacity is essential to obtain enough signal from the sequencing reactions.

The sequence information obtained by the described assay can be used for many applications. The primers can be designed to examine biologically, immunologically, or clinically interesting regions of the genome of the pathogen. In parallel assays, different parts of the genome can be examined and thus make the gathering of structural information from different regions of the genome possible. This sequence information can be used simply to confirm that the sample indeed contains the specific target DNA. In addition, a large amount of information of epidemiological relevance can be gathered to allow both geographical and historical comparisons. Furthermore, the variations in immunologically relevant proteins, such as neutralizing viral antigens, can be determined with the assay described here by sequencing the corresponding gene. Finally, it might be possible to determine the occurrence of drug resistance or tolerance in certain viral or bacterial pathogens during therapeutical therapy.

The fact that the DIANA assay and most PCR-based assays are purely qualitative limits the system to diagnosis of diseases where only the presence or absence of a pathogen is relevant. However, for many diseases it is essential also to quantitate the pathogen. Recently, we have modified the *lac* operator-based system described here to allow simple quantitation of the specific target DNA after PCR amplification (M.U., J.L., and J.W., unpublished data). Such quantitative methods might be useful for diagnosis of many diseases where the amount of pathogen must be determined. The sensitivity of the assay has not been investigated in this paper because reliable quantitative standard assays are lacking. However, using malaria-specific primers, we have recently shown that approximately 10 malaria parasites can be reproducibly detected with the DIANA procedure (M. Holmberg, J.W., J.L., and M.U., unpublished data).

In conclusion, a rapid colorimetric DIANA assay is described that is well suited to clinical diagnosis. For routine analysis, a positive control must be run in parallel to demonstrate that the amplification efficiencies have not been reduced because of the presence of inhibitors in the sample. This can be performed by amplifying a host-specific sequence in a separate assay. It is noteworthy that the introduction of the *lac* operator into the target DNA allows solid-phase sequencing with a general "universal" primer corresponding to the *lac* operator sequence. Here, this general sequencing primer was used to demonstrate that the sequence of all the positive chlamydial samples have mutations in the target gene as compared with the published sequence.

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