Urol Res (1996) 24:107–111

ORIGINAL PAPER

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Polymerase chain reaction in the diagnosis of urinary tract tuberculosis

Received: 29 December 1994 / Accepted: 15 August 1995

Abstract The polymerase chain reaction (PCR) is a technique that can be used to amplify a specific DNA genomic sequence, whereby the presence of an extremely small number of bacteria can be detected. The high sensitivity of PCR is particularly useful in paucibacillary situations such as non-pulmonary tuberculosis (TB). The aims of the present study were to establish a PCR assay for the rapid detection of *Mycobacterium tuberculosis* (MTb) in urine, to compare the sensitivity of PCR with routine culture technique (Bactec) and to determine the optimal type of urine specimen for PCR detection of MTb. In the first phase of the study, a total of 92 urine specimens were collected from 83 patients with suspected urinary tract TB. Two urine specimens in 2 patients were positive for TB by both PCR and Bactec, while 90 specimens from 81 patients were negative by both methods. Inhibition of PCR was present in nine urine specimens (10%). In the second phase of the study, a further seven patients were selected for intensive investigation to determine the optimal urine sampling for PCR detection of MTb. The conclusions of the study are that PCR can provide much faster confirmation of urinary TB (within 24–48 h) than Bactec urine culture (which may take several weeks). About 10% of urine specimens could not be evaluated by PCR due to the presence of inhibitory substances of unknown nature. MTb organisms were found to be excreted intermittently in the urine of infected patients, and single specimens were more likely to be false negative than a 24-h sample. The best method appeared to be the concentration of a large volume of urine, for instance 1 l concentrated to 2 ml.

Key words Polymerase chain reaction · Tuberculosis · Urinary tract infection

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The confirmation of urinary tract tuberculosis (TB) requires the microscopic identification [by Ziehl-Nielsen (ZN) stain] or culture of *Mycobacterium tuberculosis* (MTb). Smear examination is insensitive, requiring a minimum of about 10^4 organisms/ml for detectability [12]. The culture of MTb may take as long as 10 weeks on solid medium, and 10–20 days by the radiometric Bactec system, so that clinical and therapeutic decisions often have to be made before the laboratory diagnosis becomes available [1, 2, 4, 5]. The laboratory culture of MTb is relatively sensitive (10–100 viable organisms/sample), but may be false negative when clinical samples contain only a few, or non-viable organisms [1, 5].

The polymerase chain reaction (PCR) is a technique that can be used to amplify extremely small amounts of a specific DNA genomic sequence, whereby the presence of an extremely small number of bacteria can be detected within 24–48 h [1]. PCR has been used successfully to identify the presence of MTb in sputum specimens within 48 h [1]. The limit of detectability of PCR may vary from about ten organisms to, theoretically, as little as one bacillus (1 fg DNA/PCR vial – one mycobacterium contains approximately 5 fg DNA) [6, 11, 12, 14]. The high sensitivity of PCR is particularly useful in paucibacillary situations, especially non-pulmonary tuberculosis [12]. PCR has not been extensively tested in low-prevalence situation, such as community screening, and it has been suggested that its negative predictive value is not yet adequate for this use [14].

The aims of our study were twofold. The first objective was to establish a PCR assay for the rapid detection of MTb in urine, and to compare the sensitivity of PCR with routine culture technique (Bactec). The second objective was to determine the optimal type of urine specimen for PCR, e.g. whether a single specimen would be as good as a 24-h urine collection, and whether a random urine sample would be as good as an early morning specimen.

Patients and methods

Clinical specimens

In the first phase of the study, a total of 92 urine specimens were collected from 83 patients with suspected urinary tract TB. This was based mainly on the symptoms, urinalysis and a "high index of suspicion". Each urine specimen was divided into two equal parts, of which one was sent for Bactec TB culture, and the other was used for the PCR assay. Of the patients, 43 (52%) were male and 40 (48%) female, and the mean age was 43.5 years, ranging from 4 to 92 years.

In the second phase of the study, a further seven patients were selected. Laboratory or imaging studies gave results highly suggestive of urinary tract TB or, alternatively, the patients had a confirmed positive urine culture for TB. Multiple urine samples were collected from these patients for analysis by PCR as well as Bactec, to determine the optimum type of urine specimen for establishing the diagnosis of TB.

Preparation of samples

Urine samples were centrifuged at 4 °C for 20 min at 4000 × g (Sorvall RC-5B centrifuge), after which the supernatant was discarded and the pellet resuspended in 200 µl TRIS buffer (0.01 M, pH 8.3). Where a 24-h urine specimen was collected a 5-ml aliquot was used for PCR and 5 ml was sent for culture. One liter of the 24-h urine specimen was then concentrated by centrifugation and the pellet resuspended in 2 ml TRIS buffer, which was used for PCR amplification and Bactec culture. Prior to amplification the samples were heated at 95 °C for 10 min. To avoid false-positive PCR results, previously described laboratory methodology was applied [16]. PCR and Bactec culture were done in separate laboratories and a sample of the TRIS buffer was included in each batch for PCR analysis to rule out sample to sample contamination.

PCR amplification

Five millilitres of the boiled sample was used for PCR amplification for 35 cycles using the method and primers described by Eisenach et al. [8]. A negative control containing all the reagents except the sample, as well as a positive control containing MTb (10^3 organisms/ml), was included in every batch. Detection of the amplified DNA product was done on a 10% polyacrylamide gel using an ethidium bromide stain and photographed on a 320-nm ultraviolet transilluminator. A 123-basepair product was regarded as a positive result.

To rule out inhibition of the amplification process, the PCR was repeated for all negative samples using the procedure described above, except that forward sequence (FS) and reverse sequence (RS) 20-mer primers were used to amplify a 266-bp DNA fragment (clone C21) cloned into the multiple cloning site of M13 mp8 [13]. Fourteen picograms of M13 template and 0.4 µM of each primer (FSP and RSP) were used in a standard PCR reaction. If the PCR with TB-specific primers was negative and the assay using the M13 cloning vector was negative, inhibition of the PCR had occurred as a result of some inhibitory substance(s) in the urine sample.

Results

In the first phase of the study 92 urine specimens from 83 patients were sent for both Bactec culture and PCR. The clinical findings on urine microscopy and culture, excretory urography (intravenous pyelography, IVP) and cystoscopy in these patients are shown in Tables 1–3. Histopathological examination confirmed the presence of TB in one bladder, one kidney and one prostate biopsy, and these biopsies came from the two patients with TB confirmed on Bactec urine culture (Table 4).

In 81 patients the urine samples were negative for TB on Bactec culture as well as PCR, and in 2 patients the urine was positive on culture as well as PCR (Table 5). Two urine specimens in 2 patients were positive for TB by both PCR and Bactec, while 90 specimens were negative by both methods (Table 5). Inhibition of PCR was present in nine urine specimens (10%).

In the second phase of the study, a group of seven patients with confirmed or strongly suspected urinary tract TB were selected for intensive study to determine the optimum type of urine specimen for PCR diagnosis of TB (Table 5).

1. Patient L.T. In this 21-year-old man with sterile pyuria the IVP showed a non-functioning right upper pole with dilated calyces and infundibular stenosis, while the remainder of the calyces were irregular and the renal pelvis was stenotic. Cystoscopy showed a haemorrhagic urothelium, and Bactec urine culture was positive for MTb in two of two early morning specimens. On 2 consecutive days urine was collected as a seperate specimen every time the patient passed urine. On day 1 PCR was positive in 14 of 15 specimens. However, on day 2 PCR was positive in only 1 of 12 urine specimens. The 24-h urine was pooled, mixed and 5-ml aliquots sent for PCR and Bactec culture. On day 1 this was positive on both culture and PCR, but on day 2 it was negative on culture and positive on PCR. When the total 24-h urine sample was concentrated to 2 ml, the PCR was positive on both days. This indicates that the organisms are excreted intermittently in the urine, and that even PCR may be false negative when only a single specimen is collected randomly.

2. Patient E.S. In this 48-year-old female with loin pain, microscopic haematuria and sterile pyuria the IVP showed severe left hydro-ureteronephrosis, and on cystoscopy there was a sessile, haemorrhagic mass around the left ureteric orifice. Histology of the biopsies showed granulomata with Langhans' giant cells, but

Table 1 Findings on urinalysis

Urinalysis	Patients (*n*)	Patients (%)
Red cells plus white cells	30	36
Red cells only	17	21
White cells only	15	18
Routine culture negative	68	82
Routine culture positive	15	18

Table 2 Findings on excretory urography (IVP)

	Patients (*n*)	Patients (%)
IVP performed	76	92
IVP normal	31	41
Blunted/dilated calyces	18	24
Cavitation/calyceal diverticulum	9	12
Hydro-ureteronephrosis	7	9
Papillary necrosis	5	7
Calculi	5	7
Nonfunctioning kidney	3	4
Small bladder	2	3

Table 3 Findings on cystoscopy

	Patients (*n*)	Patients (%)
Cystoscopy performed	56	68
Cystoscopy normal	28	50
Erythema/oedema of bladder wall	8	
Cystitis (follicular, haemorrhagic)	5	
Trabeculation	6	
Leukoplakia	2	
Urethral stricture	2	

Table 4 Findings on histopathology [obtained in 27 (33%) patients]

		Patients (*n*)
Bladder:	Chronic non-specific cystitis	12
	Cystitis with squamous metaplasia	6
	Cystitis glandularis	2
	Tuberculosis	1
	Normal	1
Kidney:	Chronic pyelonephritis	3
	Tuberculosis	1
	Renal cell carcinoma	1
	Normal	1
Prostate:	Normal	2
	Tuberculosis	1
	Benign prostatic hypertrophy	1
	Chronic non-specific prostatitis	1

Table 5 Summary of findings on Bactec culture and PCR of urine specimens

	Culture	PCR
Negative (no. of patients)	81	81
Positive (no. of patients)	2	2
Negative (no. of specimens)	90	90
Positive (no. of specimens)	2	2

1. Patient L.T. Urine culture positive for TB
Consecutive single urine specimens for 24 h:
Day 1: PCR positive in 14 of 15 specimens
Day 2: PCR positive in 1 of 12 specimens
24-h urine pooled, 5-ml aliquot taken:
Day 1: culture positive, PCR positive
Day 2: culture negative, PCR positive
1 l urine concentrated to 2 ml:
Day 1: PCR positive
Day 2: PCR positive

2. Patients E.S. Urine culture and bladder histology positive for TB
24-hour urine pooled, 5-ml aliquot taken:
Culture negative, PCR positive
1 l urine concentrated to 2 ml:
PCR positive

3. Patient J.S. Urine culture positive for TB
24-hour urine pooled, 5-ml aliquot taken:
Culture positive, PCR positive
1 l urine concentrated to 2 ml:
PCR positive

4. Patient J.V. Urine culture positive for TB
24-hour urine pooled, 5-ml aliquot taken:
PCR positive

5. Patient B.B. IVP and histology suggestive of TB
Consecutive single urine specimens for 24 h:
PCR positive in 0 of 23 specimens
24-h urine pooled, 5-ml aliquot taken:
Culture negative, PCR negative
1 l urine concentrated to 2 ml:
PCR positive

6. Patient F.F. Urine culture positive for TB, on anti-TB treatment for 3 weeks
Day 1: PCR positive in 0 of 11 specimens
Day 2: PCR positive in 0 of 10 specimens
24-hour urine pooled, 5-ml aliquot taken:
Day 1: culture negative, PCR negative
Day 2: culture negative, PCR positive
1 l urine concentrated to 2 ml:
Day 1: PCR positive
Day 2: PCR positive

7. Patient W.P. Imaging suggestive, but urine culture and bladder histology not suggestive of TB
24-hour urine pooled, 5-ml aliquot taken:
Culture negative, PCR negative
1 l urine concentrated to 2 ml:
PCR negative

acid-fast organisms were not seen. Bactec culture of a single urine specimen was positive. Three days later, a 24-h urine specimen was collected, and a 5-ml aliquot was TB culture negative, but PCR positive. The concentrated 2-ml sample prepared from 1000 ml urine was also PCR positive. This indicates that an aliquot of 24-h urine may be false negative on culture, probably due to dilution, while the PCR is positive.

3. Patient J.S. In this 46-year-old female with microscopic haematuria and sterile pyuria, the IVP showed right hydro-ureteronephrosis, on cystoscopy the urothelium was hyperaemic, and Bactec urine culture was positive. A 5-ml aliquot taken from 4000 ml 24-h urine was positive on both culture and PCR, and 1000 ml urine concentrated to 2 ml was also PCR positive.

4. Patient J.V. In this 48-year-old man with acute pyelonephritis, microscopic haematuria and pyuria, erythrocyte sedimentation rate (ESR) was 60 and the IVP showed features suggestive of TB of the L1-2 vertebrae, with a non-functioning right kidney, and a single urine specimen was TB positive on Bactec culture. A 5-ml aliquot of 24-h urine was PCR positive.

5. Patient B.B. In this 33-year-old female with cystitis symptoms and sterile urine culture, the IVP showed bilateral hydro-ureteronephrosis (more severe on the left) with a small bladder, and on cystoscopy there was diffuse erythema, while histology of the biopsies showed a non-specific chronic inflammatory reaction. Separate urine specimens were collected every time the patient passed urine for 24 h, and PCR was negative in all 23 samples collected. The 24-h urine sample was pooled, mixed and 5-ml aliquots were negative for both Bactec culture and PCR. When 1000 ml urine was concentrated to 2 ml, it was PCR positive.

6. Patient F.F. In this 48-year-old female with asymptomatic microscopic haematuria the IVP showed dilatation and straightening of the ureter, the ESR was 68 and Bactec urine culture was positive. After 3 weeks on treatment with Rifater (rifampicin, isoniazid and pyrazinamide) separate urine specimens were collected for 24 h. PCR was negative in all 11 samples on day 1, and in all 10 specimens on day 2. When the 24-h urine from days 1 and 2 was pooled separately and a 5-ml aliquot was taken, culture was negative on both days, but PCR was negative on day 1 and positive on day 2. When 1000 ml of the 24-h urine from each day was concentrated to 2 ml, the PCR was positive on both days. This indicates that even after 3 weeks of treatment against TB, when the Bactec culture is negative, PCR can detect organisms in the urine, although it is not possible to say whether these are viable or dead organisms. These findings also indicate that when a large volume of urine is concentrated, the sensitivity of PCR is increased.

7. Patient W.P. In this 32-year-old male with organic brain syndrome due to alcohol abuse, urinary incontinence and sterile pyuria, two urine specimens were TB culture negative. The IVP showed hydro-ureteronephrosis on the right and non-function on the left, with a small bladder. Video-cystometrography showed a small-capacity, high-pressure bladder with vesico-ureteric reflux on the right, cystoscopy showed a dilated posterior urethra with severe bladder trabeculation, and histology of bladder biopsies showed some dystrophic calcification. A 5-ml aliquot of 24-h urine was Bactec culture and PCR negative, and the 24-h urine concentrated to 2 ml was also PCR negative.

Discussion

In the analysis of sputum samples, PCR has shown a sensitivity of 98.5% and specificity of 88.2% [14]. In the analysis of other biological samples, the sensitivity of PCR was 97%, with a specificity of 76% [4].

False-negative results with PCR may result from (1) the presence of inhibitors; (2) non-homogeneous distribution of bacteria in the specimen so that the fraction tested does not contain mycobacteria; or (3) low numbers of mycobacteria in the specimen, which decreases the probability of presence of organisms in the fraction analysed by PCR [2]. Measures to minimize the frequency of false-negative results include testing several specimens from each patient, choosing the most appropriate and good-quality specimens, removing inhibitors of PCR and concentrating specimens before analysis [2, 15]. It has also been suggested that sonication of clinical specimens may be an effective method for releasing mycobacterial DNA for amplification by PCR [3].

The PCR is essentially an enzymatic reaction, and may be susceptible to interference by metabolites, drugs (especially nucleoside analogues) or other biological substances found in the body fluids [9]. Urine is notorious for containing enzyme inhibitors, which is not unexpected, since it is the main vehicle for excretion of breakdown products and drugs from the body [7, 9, 10].

In a report of 514 specimens analysed for MTb by PCR, inhibitors of the amplification reaction were present in 25 (5%). The presence of inhibitors varied from 17% in bone marrow and 16% in blood samples, to 4.6% in urine and 3.2% in sputum samples [2]. It has been suggested that inhibition could result from residual traces of haemoglobin, sodium dodecyl sulphate or phenol, which are known to be potent inhibitors of the *Taq* polymerase [2]. In a study of PCR for cytomegalovirus DNA, it was found that urea was the predominant inhibitory constituent of urine [9]. Pretreatment of samples with guanidium thiocyanate may reduce the proportion of false-negative results and of samples that contain inhibitors [2]. Sucrose treatment of sputum samples has been found to remove most of the *Taq* polymerase inhibitors [14]. Prior dilution of the urine to reduce the urea concentration may be effective, but reduces the sensitivity of the technique [10]. Ultrafiltration is a simple and effective method for the removal of low molecular weight inhibitors [10]. It is possible that use of a smaller volume of urine for PCR assay may reduce the inhibitory effect while retaining sufficient sensitivity [7].

False-positive results with PCR may be caused by contamination due to the presence of amplicons or MTb complex bacilli or DNA [11]. Negative controls run with each batch of samples were used in this study to identify any contaminating DNA. False positives can also be caused by using too many amplification cycles or an annealing temperature which may be too low to allow specific amplification [4].

It has been shown that samples from patients being treated for TB can be positive for PCR but negative in culture, which is consistent with the fact that treated patients can harbour mycobacteria several months after cultures have become negative [1].

The conclusions of the present study are that PCR provides much faster confirmation of urinary TB (within 24–48 h) than Bactec urine culture (which takes several weeks). PCR appears to be more sensitive than Bactec culture in the diagnosis of urinary tract tuberculosis, but the true sensitivity and specificity of PCR for urinary TB needs to be evaluated in a larger group of patients. About 10% of urine specimens contained inhibitory substances, and thus could not be evaluated by PCR. The precise nature of the inhibitory substances remains unknown. A reliable method for the elimination of urinary substances which inhibit PCR is required to increase the usefulness of this technique for rapid diagnosis of urinary TB. Monitoring of false-negative results with the inclusion of an internal control DNA fragment that will amplify with the same set of primers will also facilitate the detection of urinary TB by PCR amplification [9]. The organisms appear to be excreted intermittently in the urine, and single specimens are more likely to be false negative than the 24-h sample. The best method appears to be the concentration of a large volume of urine, for instance 1 l concentrated to 2 ml.

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