JOURNAL OF CLINICAL MICROBIOLOGY, June 1997, p. 1571–1574 Vol. 35, No. 6
0095-1137/97/$04.00+0

Detection of Hydatid Antigen in Urine by Countercurrent Immunoelectrophoresis

S. C. PARIJA,[1]* P. T. RAVINDER,[1]† AND K. S. V. K. SUBBA RAO[2]

Departments of Microbiology[1] and Cardio-Thoracic Surgery,[2] Jawaharlal Institute of Postgraduate Medical Education and Research, Pondicherry, India

Received 15 November 1996/Returned for modification 10 January 1997/Accepted 11 March 1997

Hydatid antigen was demonstrated for the first time in the urine of patients with hydatid disease by countercurrent immunoelectrophoresis (CIEP). The antigen was detected in the concentrated urine of 7 of 16 (43.75% positive) patients with surgically confirmed hydatid disease, 4 of 10 (40% positive) patients with ultrasound-proven hydatid disease (daughter cysts or prominent septation and hydatid sands demonstrated by ultrasound), and 8 of 14 (57.14% positive) patients with clinically diagnosed (presumptive) hydatid disease. No antigen was detected in the concentrated urine from 24 patients with parasitic diseases other than hydatid disease. However, antigen was detected in 2 (8% false positive) of 25 concentrated urine samples collected from healthy control subjects (blood donors and students). These result suggest that the detection of hydatid antigen in the urine by CIEP is a simple, rapid, and noninvasive method of diagnosis of hydatid disease.

The development of diagnostic techniques for hydatid disease that can be used under field conditions is a priority (11). The diagnostic methods in hydatid disease essentially include the immunodiagnostic methods which demonstrate either circulating hydatid antibodies or antigens in the serum. However, collection of the blood for serum is an invasive procedure requiring technical expertise and disposable syringes to prevent the risk of transmission of serious infectious agents such as human immunodeficiency virus and hepatitis B virus. Noninvasive methods would therefore be of immense value in the diagnosis of cases of hydatid disease and in epidemiological studies of hydatid disease. Therefore, emphasis has recently been placed on the demonstration of microbial antigens in various body fluids other than serum such as saliva, sputum, and urine (1, 6). Demonstration of antigen in the urine is suggested to be of much value, because the collection of urine is a safe and noninvasive procedure and the urine can be collected easily and frequently without causing any inconvenience to the patient.

Excreted microbial antigens have been demonstrated in the urine of patients with a variety of parasitic infections such as leishmaniasis (10), Chagas' disease (7), filariasis (16), and malaria (9). In patients with hydatid disease, the circulating hydatid antigens have been demonstrated in the serum (9). It is therefore believed that the same hydatid antigen excreted in the urine could be detected. Until now, however, no such reports have been available on the detection of hydatid antigen in urine.

Here we report for the first time the development and evaluation of a countercurrent immunoelectrophoresis (CIEP) test for the detection of excreted hydatid antigen in urine. CIEP is widely used as a simple and rapid immunoassay for the detection of antibodies as well as antigens in the serum of patients with a variety of microbial infections including hydatid disease (14).

* Corresponding author. Present address: Department of Microbiology, Clinical Laboratory Services, B. P. Koirala Institute of Health Sciences, Dharan, Nepal. Fax: 977-25-20251. E-mail: bpkihs@npl.healthnet.org.

† Present address: Department of Microbiology, Kilpauk Medical College, Kilpauk, Madras, India.

In the present study, CIEP is based on the principle that wells containing hyperimmune hydatid antisera are placed on the anodic side of the electrophoretic chamber and the urine to be tested for hydatid antigen is placed on the cathodic side. If the urine contains hydatid antigen, during the process of electrophoresis in an electric field, the hydatid antigen and antibodies are driven toward each other and form a line of precipitation. A positive reaction is identified by the lines of precipitation between hyperimmune serum and hydatid antigen in the urine.

MATERIALS AND METHODS

Urine. Urine specimens were collected from patients attending the Jawaharlal Institute of Postgraduate Medical Education and Research Hospital, Pondicherry, India. These included 16 specimens from patients with surgically proven hydatid disease and 10 specimens from patients with ultrasound-proven hydatid disease. The cysts which showed daughter cysts or prominent septation and pathognomonic hydatid sands in cysts by ultrasound were diagnosed as ultrasound-proven cases of hydatid disease. This study also included 14 specimens from patients with clinically diagnosed (presumptive) hydatid disease. In the latter group, the patients presented with clinical signs and symptoms of hydatid disease, but were not operated on; hence the diagnosis could not be proved by surgery. In addition to these patients, urine specimens were collected from 49 negative control subjects (24 from patients with parasitic infections and miscellaneous conditions and 25 from healthy controls, which consisted of blood donors and student volunteers who had not suffered from hydatid disease in the recent past).

A total of 5 ml of urine was collected from each patient in sterile glass vials by aseptic technique, and specimens were labelled and stored at −20°C until use.

Concentration of urine. The urine was concentrated by the method of ethanol precipitation described by Doskeland and Berdal (6). One milliliter of urine was mixed with 0.1 ml of a cold solution of 3 M sodium acetate, 0.1% bovine serum albumin, and 0.1% (wt/vol) sodium azide. To this solution, 1.5 ml of 96% (vol/vol) ethanol stored at 20°C was added. The mixture was blended in a vortex mixture and was centrifuged at 10,000 × *g* for 10 min at 4°C. The supernatant was discarded, and the concentrated pellet of urine was resuspended in 0.1 ml of phosphate-buffered saline (pH 7.2).

Both normal (unconcentrated) and concentrated urine specimens from each patient were tested in parallel for hydatid antigen by CIEP.

Hyperimmune antiserum. Sterile human hydatid cyst fluid was emulsified with an equal volume of Freund's complete adjuvant. Adult rabbits (weight, 3 to 4 kg) were immunized by intramuscular inoculation of 0.5 ml of emulsion into each of the four limbs. After 6 weeks, 0.5 ml of the same antigen, but in Freund's incomplete adjuvant, was reinjected intramuscularly into each of the four limbs. Ten days after the final inoculation, the rabbits were bled and the blood samples were monitored for antibodies to human hydatid cyst fluid by an indirect hemagglutination (IHA) test (12). The IHA test antibody titer was 1 in 1,024.

Hyperimmune antiserum containing hydatid antibodies was purified by the method described by Gottstein (8). Briefly, 1 ml of cold serum was mixed with 1

TABLE 1. Evaluation of immunoassays for the diagnosis of hydatid disease

Subject group	No. of subjects	No. (%) of patients positive by the following:			
		IHA test	CIEP		
				Urine antigen	
		Serum antibody	Serum antigen	Unconcentrated urine	Concentrated urine
Surgically confirmed hydatid disease	16	11 (68.75)	8 (50)	4 (25)	7 (43.75)
Ultrasound-proven hydatid disease	10	7 (70)	4 (40)	2 (20)	4 (40)
Presumptive hydatid disease	14	10 (71.42)	6 (42.85)	3 (21.42)	8 (57.14)
Controls with other parasitic diseases	24	3 (12.5)	0 (0)	2 (8.33)	0 (0)
Healthy controls	25	0 (0)	0 (0)	0 (0)	2 (8)

ml of cold saline at pH 7. The serum-saline mixture (2 ml) was added to 2 ml of 50% cold saturated ammonium sulfate (pH 7) dropwise while stirring for 30 min on ice and was then centrifuged (3,000 rpm at 0°C) in a cold centrifuge. The supernatant was discarded, the precipitate was suspended in 2 ml of saline, and the procedure was repeated until the supernatant was colorless. The final precipitate was suspended in 1 ml of saline and was dialyzed against phosphate-buffered saline (pH 7.2) to remove all the residual ammonium sulfate. The titer of the purified antiserum was 1 in 2,048 by the IHA test.

CIEP. CIEP was performed by the method described by Shariff and Parija (14). Briefly, a gel was made on a glass slide by using 1% Difco Bacto Agar. It was allowed to set at room temperature and thereafter was stored at 4°C overnight before use.

CIEP was performed on the gel slides. Parallel rows of wells (4 mm in diameter and 3 mm apart) were punched out on the slides with the aid of a template. Six pairs of wells were punched in each slide. Wells containing hyperimmune antisera (titer, 1 in 2,048) were placed on the anodic side of the electrophoretic chamber, and those containing the urine to be tested were placed on the cathodic side. Ten microliters of urine was put in the well for detection of hydatid antigen, and then a current of 8 V/cm was applied for 30 min. The slides were read unstained and also after staining with amido black.

A positive reaction was defined by a precipitation line between the side with hydatid antigen in the urine and the side with hyperimmune serum. Positive and negative controls were included with each test.

Washing and staining of slides were carried out by immersing the slides for 24 h in Veronal buffer. The slides were then washed for 15 min in a 1% solution of amido black made in a solvent containing 1 ml of glacial acetic acid, 49 ml of distilled water, and 50 ml of methylated spirit for 30 min. Excess stain was washed with the solvent to give a preparation with dark bluish precipitation bands on a clear background. The slide was finally soaked in 1% acetic acid containing 1% glycerol for 15 min and was dried at 37°C in an incubator.

Serum. Serum samples were collected from all patients and control subjects. Hydatid antibodies in the sera were detected by the IHA test with double aldehyde-stabilized human O cells sensitized with the optimum sensitizing dose of hydatid antigen (12). Hydatid antigen in the sera was detected by CIEP by the method that we described earlier (14). The test was performed to detect hydatid antigen in the serum in the same way as described above for the urine antigen, except that serum instead of urine was used in the cathode wells.

RESULTS

In a positive CIEP test, the presence of hydatid antigen in urine is detected as a line of precipitation between two wells, with one containing the test urine and the other containing raised hyperimmune antisera rich in hydatid antibodies. In a negative test, the lines of precipitation were absent for urine negative for the hydatid antigen.

Hydatid antigens in the urine were detected in the unconcentrated urine of four (25%) patients with surgically confirmed hydatid disease, two (20%) patients with ultrasound-proven hydatid disease, and three (21.42%) patients with clinically diagnosed cases of hydatid disease. The antigens were also detected in the unconcentrated urine of 2 (8.33%) controls with parasitic diseases but were absent from the urine of healthy controls.

After concentration of the urine by ethanol precipitation, the antigen was detected in the concentrated urine of seven (43.75%) patients with surgically confirmed hydatid disease, four (40%) patients with ultrasound-proven hydatid disease, and eight (57.14%) patients with clinically diagnosed cases of hydatid disease. The antigens were detected in the urine of two (8%) healthy controls. Antigens were not detected in the urine of any of the control patients with parasitic diseases.

Tables 1 and 2 present a comparison of the sensitivity and specificity of the IHA test for demonstration of antibodies in serum and CIEP for the detection of antigen in the serum and the detection of antigen in both unconcentrated and concentrated urine for the diagnosis of hydatid disease. Table 3 presents a comparison of the sensitivity of antigen detection in serum and urine of the different patients with hydatid disease and the controls by CIEP.

DISCUSSION

CIEP is a simple, inexpensive, and rapid test. In this laboratory, CIEP was standardized and evaluated earlier to demonstrate circulating hydatid antigen in serum for the diagnosis of hydatid disease (14).

The present study shows for the first time the detection of hydatid antigen in urine by CIEP. In our study, CIEP detected antigen in the unconcentrated urine of four (25%) patients with surgically confirmed hydatid disease, two (20%) patients with ultrasound-proven hydatid disease and three (21.42%) patients with clinically diagnosed hydatid disease. The sensitivity of CIEP for the detection of hydatid antigen in unconcentrated urine was very low compared to that of detection of antigen in the serum (Tables 1 and 2). The lower sensitivity of CIEP for the detection of antigen in urine in comparison to that in serum is possibly due to the lower quantity of hydatid antigen excreted in a large volume of urine. In several studies carried out on the detection of antigen in the urine for diagnosis of leishmaniasis, Chagas' disease, malaria, filariasis, etc., (7, 9, 10, 16) the urine samples have been concentrated with the aim of concentrating and detecting antigen in an increased number of urine specimens. Therefore, in the present study, an

TABLE 2. Sensitivity, specificity, and positive predictive value of immunoassays for the diagnosis of hydatid disease

Test	Sensitivity (%)	Specificity (%)	Positive predictive value
IHA test			
Serum antibody	70	93.87	90.32
CIEP test			
Serum antigen	45	100	100
Urine antigen			
Unconcentrated urine	22.5	95.91	81.81
Concentrated urine	47.5	95.91	90.47

TABLE 3. Evaluation of detection of hydatid antigen in the urine and serum by CIEP for the diagnosis of hydatid disease

Subject group	Total no. of subjects	No. (%) of subjects			
		Both urine and serum positive for antigen	Serum positive but urine negative for antigen	Urine positive but serum negative for antigen	Both urine and serum negative for antigen
Surgically confirmed hydatid disease	16	3 (18.75)	5 (31.25)	4 (25)	4 (25)
Ultrasound-proven hydatid disease	10	1 (10)	3 (30)	4 (40)	2 (20)
Presumptive hydatid disease	14	5 (35.71)	1 (7.14)	3 (21.42)	5 (35.71)
Controls with other parasitic diseases	24	0 (0)	0 (0)	0 (0)	24 (100)
Healthy controls	25	0 (0)	0 (0)	2 (8)	23 (92)

attempt was also made to detect hydatid antigen in urine after concentration.

Various methods have been followed for concentrating urine for the detection of microbial antigens. These include concentration of urine by membrane filtration (7), dialysis (10), ultrafiltration (9), and ethanol precipitation (6). In this study, we have used the ethanol precipitation method (6) because it has been found to be relatively simple and the reagents and chemicals are easily and readily available. In the present study, the ethanol precipitation method successfully concentrated the hydatid antigen in urine. The CIEP could detect antigen in concentrated urine of additional patients with hydatid disease. The assay detected antigen in the concentrated urine of 7 of 16 (43.8% positive) patients with surgically confirmed disease, 4 of 10 (40% positive) patients with ultrasound-proven disease, and 8 of 14 (57.14% positive) patients with clinically diagnosed (presumptive) hydatid disease. The sensitivity of CIEP for the detection of antigen in concentrated urine compares well with that of CIEP for the detection of antigen in serum (Table 3). CIEP was also found to be specific. No antigen was detected in the concentrated urine from 24 control patients with parasitic diseases other than hydatid disease. The test showed a rate of false positivity of 8% by showing a positive reaction for antigen in the concentrated urine of 2 of 25 healthy controls.

CIEP diagnosed a total of 19 (47.5%) and 18 (45%) cases of hydatid disease by demonstrating antigen in the urine and serum of the patients, respectively. The results of the study (Table 3) indicate that the combined detection of antigen in both urine and serum led to the diagnosis of increased numbers of cases of hydatid disease. This leads to the suggestion that the combined detection of antigen in the urine and serum of a patient would help in the optimum diagnosis of hydatid disease. The failure of CIEP to detect antigen either in urine or in serum of patients with surgically confirmed hydatid disease and hydatid disease confirmed by other means could be due to various reasons. One reason is that the antigen volume is too low to be detected by CIEP. It is a recognized fact that although CIEP is a highly specific test, it is nevertheless only moderately sensitive. Therefore, the use of other immunoassays such as bacterial coagglutination and enzyme-linked immunosorbent assay, which are used in hydatid serology for the detection of hydatid antigen in serum (3, 15) or hydatid fluid (4, 13), may be evaluated to detect minute volumes of antigen in urine that are not detected by CIEP. Other possibilities are that no antigen may be found in the serum and urine or that free antigens in the serum may be bound to the antibodies, thereby forming immune complexes. The level of antigen in the serum also depends upon the strain type, anatomical location of the cyst, cyst wall structure, and speed and type of growth of the hydatid cysts (2, 5). The failure of CIEP to detect antigen in the urine of the same patient whose serum was positive for antigen by the test is possibly due to very low level of antigenuria in comparison to the level of antigenemia. The absence of antigen from serum but the presence of antigen in the urine of the same patient, as seen in some patients with hydatid disease, is difficult to explain (Table 3). This could possibly be due to a high concentration of antigen in urine following concentration. This is supported by the fact that antigen was absent from the unconcentrated urine of all except one of the patients in the present study.

CIEP is a simple, rapid, and inexpensive test. The test has also been successfully used to detect *Leishmania donovani* antigen in the urine of patients with leishmaniasis (10). The results of the present study indicate that CIEP is a moderately sensitive and equally specific test for the detection of hydatid antigen in the urine. It is hoped that it detects the same hydatid antigen in urine that is circulating in the serum of patients with hydatid disease. Therefore, demonstration of antigen in urine offers a noninvasive, easy, and safe alternative means of diagnosis of hydatid disease not only under field conditions but also in routine clinical laboratories. It has the potential for wider use in clinical laboratories because detection of hydatid antigen in urine, which can be more safely obtained than blood, would be immensely useful for establishing the etiological diagnosis of hydatid disease.

REFERENCES

1. **Berdal, B. P., E. F. Carol, and C. F. John.** 1979. Detection of *Legionella pneumophilia* antigen in urine by enzyme-linked immunosorbent assay. J. Clin. Microbiol. **9:**575–578.
2. **Coltorti, E. A., and V. M. Varela Dias.** 1975. Penetration of host IgG molecules into hydatid cysts. J. Parasitenkd. **48:**47–51.
3. **Craig, P. S., and G. S. Nelson.** 1984. The detection of circulating antigen in human hydatid disease. Ann. Trop. Med. Parasitol. **78:**219–227.
4. **Craig, P. S., W. Bailey, and G. S. Nelson.** 1986. A specific test for the identification of cyst fluid samples from suspected human hydatid. Trans R. Soc. Trop. Med. Hyg. **80:**256–257.
5. **D'Amellio, R., O. Pontesilli, L. Palmisano, M. Pezzells, V. Vullo, S. Delia, F. De Rose, F. Sorice, and F. Aiuti.** 1983. Detection and partial characterization of circulating immune complexes in hydatid disease. J. Clin. Microbiol. **18:**1021–1026.
6. **Doskeland, S. O., and B. P. Berdal.** 1980. Bacterial antigen detection in body fluids. Methods for rapid antigen concentration and reduction of non-specific reactions. J. Clin. Microbiol. **11:**380–384.
7. **Freilij, H. L., and R. S. Corral.** 1987. Antigenuria in infants with acute congenital Chagas' disease. J. Clin. Microbiol. **25:**133–137.
8. **Gottstein, B.** 1984. An immunoassay for detection of circulating antigen in human echinococcosis. Am. J. Trop. Med. Hyg. **33:**1105–1111.
9. **Katzin, A. M., E. S. Kincira, O. P. Claudio, M. Alexandre, and A. M. S. Valrumos.** 1991. Detection of antigen in urine of patients with acute falciparum and vivax malaria infections. Am. J. Trop. Med. Hyg. **45:**453–462.
10. **Kohanteb, J., S. M. Ardehali, and H. K. Rezai.** 1987. Detection of *Leishmania donovani* antigen and antibody in the urine of visceral leishmaniasis patients. Trans R. Soc. Trop. Med. Hyg. **81:**578–580.
11. **Parija, S. C.** 1993. Simple immunoassays in the serodiagnosis of hydatid disease in the field or poorly equipped laboratories. Indian J. Med. Microbiol. **11:**1–4. (Editorial.)
12. **Parija, S. C., and R. S. Rao.** 1986. Enhancement of sensitivity of the haemagglutination tests for echinococcus by use of *Staphylococcus aureus* protein A. J. Med. Microbiol. **22:**241–244.

13. **Parija, S. C., P. T. Ravinder, and M. Shariff.** 1996. Detection of hydatid antigen in the fluid samples from hydatid cysts by co-agglutination. Trans. R. Soc. Trop. Med. Hyg. **90:**255–256.
14. **Shariff, M., and S. C. Parija.** 1991. Counter-current immunoelectrophoresis test for serodiagnosis of hydatid disease by detection of circulating hydatid antigen. J. Microbiol. Methods **14:**71–76.
15. **Shariff, M., and S. C. Parija.** 1993. Co-agglutination (Co-A) test for circulating antigen in hydatid disease. J. Med. Microbiol. **28:**231–234.
16. **Zheng Huijun, Zhenghou Taw, M. V. R. Redddy, B. C. Harinath, and W. F. Piessens.** 1987. Parasite antigens in sera and urine of patients with Bancroftian and Brugian filariasis detected by sandwich ELISA with monoclonal antibodies. Am. J. Trop. Med. Hyg. **36:**554–560.