Rapid, Simple, and Reliable Method for the Diagnosis of CAPD Peritonitis Using the New MMP-9 Test Kit

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The objective of the present study was to evaluate the sensitivity and efficiency of the matrix metalloproteinase-9 (MMP-9) test kit for the diagnosis of bacterial peritonitis in patients undergoing peritoneal dialysis (PD). Peritoneal effluents were collected from seven continuous ambulatory PD (CAPD) patients with peritonitis, four patients with suspected peritonitis, 30 maintenance PD patients without infection, and seven patients at initiation of PD. The MMP-9 test kit was used to analyze 112 peritoneal effluent samples. These peritoneal effluents were also used to count leukocytes and examine microorganisms. MMP expression was measured by gelatin zymography, and activities were measured by an enzyme-linked immunosorbent assay (ELISA). The relationship between the

reactivity of the test kit and the number of leukocytes in the samples was examined. There was a significant difference in the number of leukocytes in peritoneal effluents between the negative and positive groups detected by the MMP-9 test kit ($P < 0.0001$). The results obtained with the MMP-9 test kit were negative for peritoneal effluent samples that did not show increased cell counts. The reactivity of the MMP-9 test kit showed no significant differences among various microorganisms, and remained stable. The MMP-9 test kit appears to be a simple and reliable method for early diagnosis of CAPD peritonitis, and reflects the leukocyte count in peritoneal effluents. *J. Clin. Lab. Anal.* 18:224–230, 2004. © 2004 Wiley-Liss, Inc.

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INTRODUCTION

Bacterial peritonitis is one of the most important complications that occur in patients undergoing peritoneal dialysis (PD). Most cases of bacterial peritonitis are improved by antibiotics; however, peritonitis in long-term PD patients may sometimes lead to ultrafiltration loss and reduced dialysis efficiency. A few patients progress to encapsulating peritoneal sclerosis (EPS). Since damage to the peritoneum by peritonitis leads to peritoneal degradation and impairment of peritoneal solute transportation, it is important to diagnose and treat peritonitis early (1–5).

Fukudome et al. (6) reported that MMP-9 activity in peritoneal effluents increases in continuous ambulatory PD (CAPD) patients with peritonitis. MMP-9 may be associated with peritoneal extracellular matrix (ECM) remodeling during peritonitis, and is expected to become

a new marker for early diagnosis in PD patients with peritonitis (6,7). Therefore, we developed a new MMP-9 test kit consisting of MMP-9 antigen conjugated with a colloidal dye produced to detect antibody in a nitrocellulose membrane (NCP) dipstick assay based on immunochromatography (8). The objective of the present study was to validate the MMP-9 test kit for the early diagnosis of bacterial peritonitis in CAPD patients.

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MATERIALS AND METHODS

Patients

A total of 112 peritoneal effluents were collected from 48 PD patients (33 males and 15 females, mean age = 50.0 ± 12.1 years (mean $\pm$ SD), range = 26–82 years). Seven patients had peritonitis, and five patients had suspected peritonitis. Twenty-nine patients were on maintenance PD without infection, and seven patients were at initiation of PD. Renal failure was caused by chronic glomerulonephritis in 38 patients, nephrosclerosis in five patients, autosomal dominant polycystic kidney in two, diabetes mellitus in one, renal malformation in one, and toxemia of pregnancy in one.

Samples

Peritoneal effluent samples were examined with the use of the MMP-9 test kit. The relationship between the reactivity of the test kit and the number of leukocytes in the samples was examined. All peritoneal effluents were also used to count the number of leukocytes and examine microorganisms. After the MMP-9 test kit examinations were performed, the samples were immediately filtered through a 0.45- μ m filter (Millipore, MA) and stored at

-80° for the determination of MMP-9 by zymography and testing, as described previously (9).

Principle and Detection Procedure of the MMP-9 Test Kit

Test kit preparation

Dipsticks were prepared from nitrocellulose membranes (NCPs), and immunochromatography was used to assess the antigen-antibody reaction of MMP-9. The MMP-9 test kit was obtained from Daiichi Fine Chemical Industries (Toyama, Japan). The MMP-9 test strip consisted of three zones containing different antibodies: 1) a conjugate zone containing colored mouse anti-MMP-9 antibody, 2) a test zone containing mouse anti-MMP-9 antibody, and 3) a control zone containing goat anti-IgG antibody.

The mouse anti-MMP-9 antibody in the conjugate zone was dissolved in the effluent and moved to the control zone through the test zone. The dissolved anti-MMP-9 antibody and fixed mouse anti-MMP-9 antibody were conjugated with MMP-9 of the effluent in the test zone. Excess dissolved anti-MMP-9 antibody was trapped with goat anti-IgG antibody in the control zone to prepare for the anti-MMP-9 antibody and anti-IgG antibody complex.

Antibody-A : mouse anti MMP-9 antibody - 1
Antibody-B : mouse anti MMP-9 antibody - 2
Antibody-C : goat anti mouse IgG antibody,
 bound to Antibody-A

Fig. 1. MMP-9 test kit preparation and principle. Dipsticks were prepared from nitrocellulose membranes. The reaction of the MMP-9 test kit preparation was based on the immunochromatography method. MMP-9 test kit specimens were obtained from Daiichi Fine Chemical Industries (Toyama, Japan).

If MMP-9 was present in the effluent, it was first conjugated with dissolved mouse anti-MMP-9 antibody in the conjugate zone. Then the MMP-9 and its antibody complex were bound to the mouse anti-MMP-9 antibody in the test zone (Fig. 1).

Next, $>80 \mu\text{L}$ of the peritoneal effluent sample were placed in a small plastic cup. The prepared dipstick was inserted into the cup, kept there for 10–15 min at room temperature, and then removed. If both the detection and control bands were colored purple-blue, the sample was recorded as positive. If the control band was colored purple-blue but the detection band was not colored, it was taken as negative. When neither band was colored, it was assumed that the test reagents were not reacting properly (Fig. 2).

Zymography

Zymography was performed with sodium dodecyl sulfate (SDS)-10% polyacrylamide gels polymerized in the presence of 0.1% gelatin, as described previously (9). After electrophoretic separation at 14–15 mA, the gels were washed twice in 2.5% (v/v) Triton X-100 to remove SDS, and then incubated in 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM CaCl_2 at 37° for 18 hr. Gels were stained with 0.2% Coomassie brilliant blue R250 in 50% methanol/10% acetic acid, and destained with methanol and acetic acid. Gelatin-degrading activities were quantified by scanning zymography by means of a computing densitometer, and expressed as arbitrary units.

Fig. 2. Precision of the MMP-9 test kit. The prepared dipstick was immersed in the effluent for 10–15 min at room temperature and then withdrawn. If both the detection and control bands turned purple-blue, the sample was considered positive. When only the control band (and not the detection band) turned purple-blue, it was taken as negative. It appears that $>100/\text{mm}^3$ leukocytes indicate a positive result with the MMP-9 test kit. The reaction of the MMP-9 test kit depended on the number of leukocytes in the peritoneal effluent.

Measurement of MMP-9 Concentrations

The levels of MMP-9 in the peritoneal effluent were determined by an enzyme-linked immunosorbent assay (ELISA). The MMP-9 ELISA kits were also obtained from Daiichi Fine Chemical Co. Ltd. (Toyama, Japan). The levels of MMP-9 ranged from 3.1 to 100 ng/ml (10).

Principle Behind Leukostix[®]

The sensitivity of Leukostix[®] (Bayer, IN) is dependent on the activity of leukocyte elastase, reaction time, and humidity. The reaction of Leukostix[®] is dependent on leukocyte elastase and diazo coupling of 4-diazo-3-hydroxy-1-naphthylsulfonate (HONapN+2) with the enzymatic hydrolysis product HOPPy (11). The use of Leukostix[®] for the diagnosis of urinary infection is well known to be related to the neutrophil count, as described previously (12–14).

Statistical Analysis

Data were expressed as mean $\pm$ SD. Correlations were determined by Spearman's correlation coefficient. The two groups were compared by means of Student's *t*-test. Statistical differences were considered significant at a *P*-value < 0.05 .

RESULTS

Relationship Between Reactivity of the MMP-9 Test Kit and Various Microorganisms in Patients With Bacterial Peritonitis

There was no significant difference in the reactivity of the MMP-9 test kit among various microorganisms (data not shown). We defined critical bacterial perito-

nititis in PD patients as leukocytes exceeding 100/mm³ and neutrophils exceeding 50% in PD effluent, with a cloudy peritoneal effluent and the patient complaining of fever and abdominal pain, as previously described (15). Bacterial peritonitis was caused by *Group β streptococcus* in one patient, *Staphylococcus epidermidis* in one, *Staphylococcus oralis* in one, *E. coli* in one, *Pseudomonas* in one, and *Corynebacterium jeikeium* in one. No microorganisms were detected in one patient. This patient fit the above-described criteria, but no microorganisms were detected in the effluent. Suspected patients had $< 100/\text{mm}^3$ leukocytes, and did not fit the bacterial peritonitis criteria.

Relationships Among the Number of Leukocytes, Reactivity of the MMP-9 Test Kit, and Levels of MMP-9 in Peritoneal Effluent

There was no correlation between the levels of MMP-9 and the number of leukocytes in the effluent at the initiation of PD (Fig. 3a). The activity of MMP-9 in peritonitis patients was significantly higher than that in noninfected controls ($P < 0.01$) (Fig. 3a). The reaction of the MMP-9 test kit depended on the number of leukocytes in the peritoneal effluent (Fig. 2). MMP-9 concentration was also dependent on the number of leukocytes in the peritoneal effluent ($r = 0.916$, $P < 0.001$; Fig. 3a). There was a significant correlation between the levels of MMP-9 and the number of neutrophils in the effluent of bacterial peritonitis PD patients ($r = 0.918$, $P < 0.001$; Fig. 3b). The number of leukocytes and neutrophils in the peritoneal effluents significantly differed between the positive and negative groups detected by the MMP-9 test kit ($P < 0.0001$, Table 1).

Fig. 3. Relationship between MMP-9 activity and the number of leukocytes in the peritoneal effluent (a), and the number of neutrophils in the peritoneal effluent (b).

TABLE 1. Relationship between number of leukocytes in peritoneal effluent and test reactivity

- **MMP-9 test kit**

Sensitivity and Specificity of the MMP-9 Test Kit

The sensitivity of the MMP-9 test kit was 96.1%, and the specificity was 89.5% for bacterial peritonitis (Table 2). The positive predictive value was 73.5%, and the negative predictive value was 98.7%.

Serial Findings Regarding the Expression of MMP-9 in Peritoneal Effluent by Zymography and the Reactivity of the MMP-9 Test Kit in a Patient With Peritonitis Caused by *Group β Streptococcus* Infection

Samples were obtained from a PD patient diagnosed with peritonitis caused by a *Group β streptococcus*

TABLE 2. Sensitivity and specificity of MMP-9 test kit

	Peritonitis present	Peritonitis absent
MMP-9 positive	25	9
MMP-9 negative	1	77

infection. The reactivity of the MMP-9 test kit, the levels of MMP-9 activity, and the numbers of leukocytes and neutrophils were evaluated in the same samples. The 93 kDa proteinase is the latent form of MMP-9 (pro-MMP-9). The density of the latent form of the MMP-9 (pro-MMP-9) band clearly increased at onset of peritonitis, and then decreased and disappeared in the recovery stage (Fig. 4). The latent form of MMP-9 (pro-MMP-9), 93 kDa proteinase, was previously measured by zymography as an expression of MMP-9 in the effluent (6).

Extended Incubations of Leukostix[®] and the MMP-9 Test Kit in a Peritonitis Sample

Extended incubations of Leukostix[®] and the MMP-9 test kit were performed in the same sample obtained from a patient with peritonitis (Fig. 5). The MMP-9 test kit usually provides results within 10 min. Since the MMP-9 test kit is based on antigen-antibody reaction, no incubation time-dependent changes occurred. While the MMP-9 test kit showed a stable reaction after 30 min, the Leukostix[®] reaction gradually changed from trace to strongly positive (Fig. 5).

DISCUSSION

Bacterial peritonitis is one of the most important complications of CAPD. Peritonitis may lead to ultrafiltration failure and insufficient elimination of solute. Since peritonitis can reduce the survival rate, it is important to diagnose and treat peritonitis early (1–5).

Since MMP-9 in the effluent showed high activity before the effluent became cloudy in the bacterial peritonitis rat model, we hypothesized that it might be possible to initiate early treatment based on an early diagnosis by MMP-9 before symptoms of peritonitis (e.g., abdominal pain and cloudy effluent) appeared. We observed that MMP-9 is related to the progression of bacteria-induced peritonitis in a rat model (data not shown). The MMP-9 test kit was convenient, and the results regarding MMP-9 activity were similar to those produced by the ELISA. In this study, the MMP-9 test kit for diagnosis of bacterial peritonitis in PD patients showed very high sensitivity and specificity. In previous studies, the sensitivity and specificity of the Leukostix[®] was 92.8% and 83.3%, respectively (16). In this study, the MMP-9 test kit had higher sensitivity and specificity

Fig. 4. The reactivity of the MMP-9 test kit and the number of leukocytes and neutrophils in the peritoneal effluent were examined in a patient with peritonitis caused by *Group β streptococcus* infection. The levels of MMP-9 expression in CAPD effluents were measured by zymography from the onset of peritonitis (day 0) until recovery.

Fig. 5. Extended incubations of Leukostix[®] and the MMP-9 test kit in the same sample obtained from a peritonitis patient. Leukostix[®] usually provides results within 2 min, and the MMP-9 test kit showed stable results after 30 min. However, the Leukostix[®] results gradually changed from trace to strongly positive.

than the Leukostix[®]. Usually MMP-9 is released by neutrophils. The reactivity of the MMP-9 test kit was not affected by the number of eosinophils or detached mesothelial cells in the PD introduction stage, but it was affected by the number of neutrophils in bacterial peritonitis. The reactivity of the MMP-9 test kit showed no significant differences among various microorganisms at the onset of peritonitis. The critical value of PD peritonitis was generally considered to be $>100/\text{mm}^3$ leukocytes in PD effluent (15). In the present study,

$>100/\text{mm}^3$ leukocytes appeared to indicate a positive result with the MMP-9 test kit. Since the MMP-9 test kit is adapted for use in bacterial peritonitis, this test kit is a reliable method for the early diagnosis of this condition in PD patients. In our hospital, we usually use Leukostix[®] in all patients undergoing PD to diagnose bacterial peritonitis (16), although a few cross reactions can occur with this test kit. Since the MMP-9 test kit is based on an antigen-antibody reaction, it has a stable reactivity. In contrast, the sensitivity of Leukostix[®] is

dependent on the activity of leukocyte elastase, reaction time, and humidity. Leukostix[®] cannot provide an accurate diagnosis after 2 min, since it might produce a nonspecific reaction after that time. In this study, the reactivity of Leukostix[®] changed gradually from trace to strongly positive by 5 min after the reaction. The MMP-9 test kit appears to be a simple and reliable method for the early diagnosis of bacterial peritonitis in PD patients, and reflects the number of leukocytes in peritoneal effluents. The MMP-9 test kit may prove to be useful for cases in which the presence of fibrin exudates precludes the counting of leukocytes in peritoneal effluents. Further clinical examinations concerning the diagnosis of bacterial peritonitis, as related to factors that influence reactivity, will play an important role in clarifying the efficiency of this MMP-9 test kit.

REFERENCES

1. Keane WF, Alexander SR, Bailie GR, et al. Peritoneal dialysis-related peritonitis treatment recommendations: 1996 update. *Perit Dial Int* 1996;16:557–573.
2. Dobbie JW. Ultrastructure and pathology of the peritoneum in peritoneal dialysis. In: Gokal R, Nolph KD, editors. *The textbook of peritoneal dialysis*. Dordrecht: Kluwer Academic Publishers; 1994. p 14–44.
3. Dobbie JW, Anderson JD, Hind C. Long-term effects of peritoneal dialysis on peritoneal morphology. *Perit Dial Int* 1994;14(suppl 3):16–20.
4. Oules R, Challah S, Brunner FP. Case-control study to determine the course of sclerosing peritoneal disease. *Nephrol Dial Transplant* 1998;3:66–69.
5. Slingeneyer A, Canoud B, Mion C. Permanent loss of ultrafiltration capacity of the peritoneum in long-term peritoneal dialysis: an epidemiological study. *Nephron* 1983;33:133–138.
6. Fukudome K, Fujimoto S, Sato Y, et al. Peritonitis increases MMP-9 activity in peritoneal effluent from CAPD patients. *Nephron* 2001;87:35–41.
7. Marshall BC, Santana A, Xu QP, et al. Metalloproteinases and tissue inhibitor of metalloproteinases in human peritoneal mesothelial cells. *J Clin Invest* 1993;91:1792–1799.
8. Ro Y, Hamada C, Tomino Y, et al. Early diagnosis of CAPD peritonitis using a new test kit for detection of matrix metalloproteinase (MMP)-9. *Perit Dial Int* 2004;24:7.
9. Kossakowska AE, Edwards DR, Lee SS, et al. Altered balance between matrix metalloproteinases and their inhibitors in experimental biliary fibrosis. *Am J Pathol* 1998;153:1895–1902.
10. Fujimoto N, Hosokawa N, Hayakawa T, et al. A one-step sandwich enzyme immunoassay for human matrix metalloproteinase 9 (92kDa gelatinase/type IV collagenase) using monoclonal antibodies. *Clin Chim Acta* 1994;231:79–88.
11. Jackson DS, Brown AD, Powers JC, et al. A kinetic study of the hydrolysis of the N-tosylalanine ester of 3-hydroxy-5-phenylpyrrole and related compounds by human leukocyte elastase. *Arch Biochem Biophys* 1995;323:108–114.
12. Skjold AC, Hugel H. A new dip-and-read test for leukocyte esterase activity in urine with the Ames Leukostix[®] reagent strip. *Clin Chem* 1985;31:993.
13. Pendergrass JH, Skjold AC, Stover LR, et al. A control system for monitoring the performance of leukocyte strip tests. *Clin Chem* 1986;32:1400–1402.
14. Scheer WD. The detection of leukocyte esterase activity in urine with a new reagent strip. *Am J Clin Pathol* 1987;87:86–93.
15. William FK, George RB, Stephen V, et al. Adult peritoneal dialysis-related peritonitis treatment recommendations: 2000 update. *Perit Dial Int* 2000;20:396–411.
16. Saito K, Hamada C, Tomino Y, et al. Early diagnosis of CAPD peritonitis by a test kit for detecting leukocyturia (Leukostix[®]). *J Jap Soc Dial Ther* 1991;24:913–917.