Chromogenic *Limulus Amoebocyte Lysate* Assay in Early Etiologic Diagnosis of Peritonitis in Patients on Continuous Ambulatory Peritoneal Dialysis

IULIANA ISVORANU[1], DANIELA RADULESCU[1,2], ILEANA PERIDE[1,2]*, ANDREI NICULAE[1,2], RUXANDRA DIANA SINESCU[3,4], IONEL ALEXANDRU CHECHERITA[1,2]

[1] "St. John" Emergency Clinical Hospital, Bucharest, Department of Nephrology and Dialysis, 13 Vitan Barzesti Road, 042122, Bucharest, Romania

[2] "Carol Davila" University of Medicine and Pharmacy Bucharest, Clinical Department No 3, 37th Dionisie Lupu Str., 020022, Bucharest, Romania

[3] "Elias" Emergency University Hospital, Department of Plastic Surgery and Reconstructive Microsurgery, 17 Marasti Blvd., 011461, Bucharest, Romania

[4] "Carol Davila" University of Medicine and Pharmacy Bucharest, Clinical Department No 11, 37th Dionisie Lupu Str., 020022, Bucharest, Romania

Although substantial progresses have been made in etiologic diagnosis of continuous ambulatory peritoneal dialysis (CAPD) peritonitis, the length of time until cultures are obtained remains unacceptable long; at least for the first 48 h, indiscriminate use of broad spectrum antibiotics is seen in clinical practice with various side effects and with high-rates of transfer on hemodialysis. Therefore, alternative methods for early etiological diagnosis are stringently needed. During a 4-years study, we conducted qualitative and quantitative chromogenic LAL (Limulus amoebocyte lysate) assay completed with β-1,3-glucans blocking-sequence on 114 peritonitis episodes in 43 CAPD patients. The results were analyzed in parallel with the cultures of dialysate effluents. Sensitivity and specificity for Gram-negative peritonitis were evaluated in both tests; a receiver operating characteristic (ROC) plot was used to analyze the cutoff value for a maximum sensitivity and specificity of quantitative test in diagnosing Gram-negative infections. Screening LAL chromogenic test had a sensitivity of 92.7% and a specificity of 90.4% in establishing diagnosis of Gram-negative peritonitis. In quantitative LAL test (completed with blocking LAL-β-1,3-glucans reaction), the ROC plot emphasized a sensitivity of 100% and a specificity of 95.89% at endotoxin levels of 0.423 EU/mL or greater. In conclusion, our study demonstrates a high sensitivity and specificity of chromogenic LAL assay for the detection of peritoneal Gram-negative peritonitis. Chromogenic LAL assay is a useful test in early diagnostic of peritonitis in CAPD patients and it should be recommended as a routine in clinical practice.

Keywords: CAPD peritonitis, Limulus amoebocyte lysate, Gram-negative bacteria, endotoxin.

Peritonitis represents the most significant complication of peritoneal dialysis (PD) and the main cause for transfer to hemodialysis (HD) [1-6]. It is also associated with increased morbidity and mortality [7-9]. Although periodic treatment guidelines are elaborated by International Society of Peritoneal Dialysis [10-12], indiscriminate use of broad-spectrum antibiotic treatment until effluent cultures are obtained is often seen in clinical practice [13] with several side-effects and with the development of antibiotic resistance [14-20]. In most cases, a minimum of 48h is needed to obtain the results of the cultures, but, in clinical practice it may take 3 – 7 days or more to obtain the results from the laboratory [21]. Gram staining of the dialysate effluent is not widely used in clinical practice because it is not available in all nephrology units; furthermore, literature data show large sensitivity variations of Gram staining, between 9 – 80% in peritoneal dialysate effluents [21]. Delaying the treatment until the cultures of dialysate effluent are obtained is dangerous. Improved diagnosis at presentation could prevent these unwanted effects and it could allow a more precise treatment.

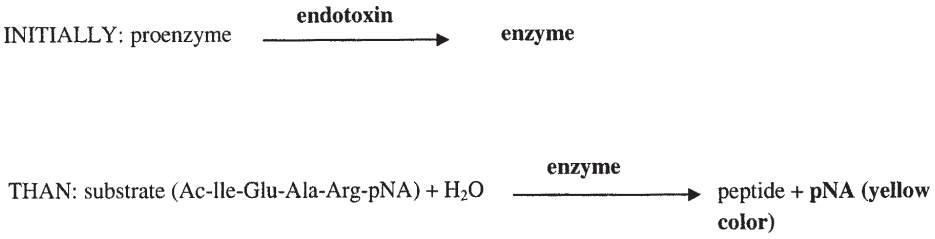
LAL assays (*Limulus amoebocyte lysate*) are the most sensitive tests currently used to detect small amounts of bacterial endotoxin (lipopolysaccharide) in various pharmaceutical products or medical devices [21,22]. They also detect β-1,3-glucans in the presence of fungus infection [23,24]. The name of the LAL test derives from the Latin name of the horseshoe crab – *Limulus polyphemus*. The LAL principle is based on an old observation which revealed that infection of this crustacean with Gram-negative bacteria, even nonviable, leads to a fatal intravascular coagulation [24-26]. This is due to an extensive clotting reaction between the endotoxin of Gram-negative bacteria and a pro-coagulant protein in the structure of Limulus amoebocytes [24, 25, 27 ,28]. The amebocytes are the crab's blood cells and LAL is an aqueous extract of these cells [22, 24, 25,29]. The reaction between the crab's pro-coagulant protein and bacterial endotoxins takes place secondary to an enzymatic mechanism [24, 25, 30-33]. There are three types of LAL test: gel-clot, turbidimetric and chromogenic [22,24,33,34]. Previously researches on different LAL assays in ameliorating early diagnosis of peritonitis in PD have reported sensitivities varying between 65 and 100% [21,24,34-39].

This prospective study was conducted to evaluate the qualitative and quantitative chromogenic LAL test significance for an early diagnosis of peritonitis in

* *email: ileana_peride@yahoo.com*

All authors had equal contribution

continuous ambulatory peritoneal dialysis (CAPD) patients. The principle of chromogenic LAL test is based on the reaction between the bacterial endotoxins and LAL reagent followed by an enzyme activation inducing yellow colour by releasing p-nitroaniline (pNA) from a synthetic substrate (Ac-lle-Glu-Ala-Arg-pNA) [25,32]:



Experimental part

Material and methods

During a 4-years period, we studied all peritonitis episodes underlined in a population of 43 ESRD undergoing CAPD. Diagnosis was established by the presence of more than 100 leukocytes/μL of PD effluent (presenting at least 50% polymorphonuclear cells) in patients having abdominal pain and cloudy peritoneal effluent. At the moment of presentation, effluent samples were collected for culture, cell count and for LAL (Limulus amoebocyte lysate) test.

The chromogenic LAL assay was performed with Lonza QCL-100™ device from Pharma & Biotech [25]. With this device, the peritoneal effluent sample is mixed with the LAL reagent in a test kit and the mixture was then incubated for 10 min at 37°C ± 1°C. In the next step, the LAL-sample is mixed with a synthetic substrate solution, then it is incubated at 37°C ± 1°C for another 6 min . The reaction is stopped with a stop reagent. If the sample contains endotoxin, then it will turn yellow; absorbance of the sample is determined spectro-photometrically at 405-410 nm. Since the absorbance is directly proportional to the amount of endotoxin contained in the test sample, the concentration of endotoxin is calculated using a standard curve. As is stipulated in the manufacturer's manual, with this device it is possible to detect even 0.1 EU/mL endotoxin values [25]. Endotoxin standard (*E. coli* 0111:B4) was used as a control [25]. In order to remove false-positive reactions due to the presence β-1,3-glucans of fungal origin [23,24], after calculating the concentration of endotoxin in the sample by graphical method, we proceeded to the test β-G-blocker that blocks the reactivity of LAL with β-1,3 glucans. This test was performed with the Kinetic-QCL device β-G-blocker from Pharma & Biotech [40].

As recommended [25,40], all samples were collected in pyrogen-free glassware with sterile pipettes, both provided from the manufacturer, and they were transported to the outside laboratory in special refrigeration boxes in maximum 1 hour within patient' presentation in case of admission before 6 pm; when the patient was admitted after 6 pm, the samples were kept in special refrigerators at -20°C until 8 am when they were transported to the laboratory. The results were obtained, depending the hour of admission, at 2 h in the earliest cases to 9 h in the latest. The results of qualitative (screening) and quantitative (endotoxin units/mL = EU/mL) were analyzed in parallel with the results of dialysate effluent cultures when they arrived from the lab. In qualitative LAL test, we expected a negative result in effluent samples with Gram positive or sterile culture; effluent samples with Gram negative or fungal culture were expected to be LAL positive. In the quantitative LAL test completed with b-G-blocker test, we analyzed the amounts of endotoxins in the effluent samples in correlation with the results of the culture.

Statistical analysis of data.

The results were expressed by central tendency indicators (for each group was calculated the mean, median, modal value and standard derivation). The comparison between groups was performed with "T-Student – Fischer" tests for quantitative variables. The results were analyzed globally, too, using analysis of variance ANOVA. Where it was considered a normal distribution, all data were expressed as mean plus standard deviation. Regarding the patients' characteristics, Mann-Whitney test was performed for continuous data and Fischer test for categorical data. Comparison between the two groups before and after treatment was performed with Student T-test. Pearson correlations were performed (Bivariate Correlations Two Tailed) to analyze associations between tested parameters. It was considered that p values < 0.05 were statistically significant. Skewness, Kurtosis and Shapiro-Wilk tests were used to analyze the distribution of data. For this, the result of the test was divided by the standard error and it was examined the range of values (the value "Z"). For values of "Z" outside the range (-1.96) – (+1.96), it was considered that the data have an irregular distribution, which required the application of Wicoxon non-parametric tests. Additionally, a statistical probability $p < 0.05$ resulting from the application of Shapiro-Wilk test made the null formulated hypothesis - namely that the data have a normal distribution – to be rejected. Histograms have a form that does not comply with normal distribution (Gauss curve). Likewise, the graphs of "Q-Q Plot" show diverted distribution from normality. Similarly, box-plot type graphs have not a symmetric distribution. In this case, the data have a significant deviation from normality which requires further using of non-parametric tests.

Results and discussions

114 episodes of peritonitis were recorded during the 4-years study, corresponding to a rate of 0.667 episodes/patient/year. Qualitative and quantitative LAL test lasted approximately 1.5h/patient. The earliest results of the cultures were obtained 4 days after the patient' admission and the latest after 14 days (in most cases negative cultures).

Screening (qualitative) LAL test was positive in 45 cases: 38 of Gram-negative peritonitis (92.68% of Gram-negative peritonitis), 4 cases with negative cultures (23.53% of culture-negative peritonitis after 14 days of incubation), and in all three fungal peritonitis (fig. 1). The episodes of peritonitis in which screening LAL test was negative presented at bacteriological analysis: Gram-negative bacteria in 3 cases (7.32% of total Gram-negative peritonitis), all 53 cases of peritonitis with Gram-positive and in 13 cases with negative cultures after 14 days of incubation (76.47% of the total culture-negative peritonitis) (fig. 2). Statistical analysis revealed that the screening

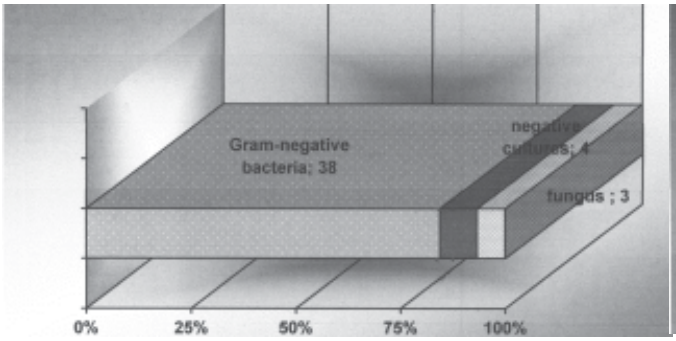


Fig. 1. Culture results in peritonitis with positive screening test LAL

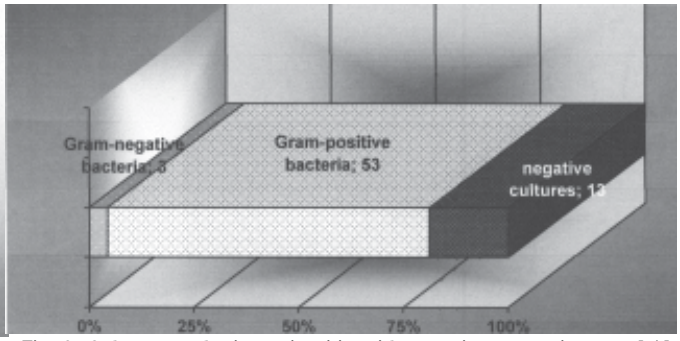


Fig. 2. Culture results in peritonitis with negative screening test LAL

		Gold Standard		Total
		Has condition	Does NOT have condition	
Test Result	*Count*	*38*	*7*	*45*
Positive	% within Test Result	84.4	15.6	100
	% within Gold Standard	**92.7**	9.6	39.5
	Count	*3*	*66*	*69*
Negative	% within Test Result	4.3	95.7	100
	% within Gold Standard	7.3	**90.4**	60.5
	Count	*41*	*73*	*114*
Total	% within Test Result	36	64	100
	% within Gold Standard	100	100	100

Table 1
SENSITIVITY AND SPECIFICITY OF SCREENING LAL TEST (TEST RESULT *VERSUS* GOLD STANDARD – CROSS TABULATION)

(qualitative) LAL test was significantly more often positive during the episodes of peritonitis caused by Gram-negative bacteria when compared with the other types of peritonitis. In this test, the sensitivity for diagnose Gram-negative peritonitis was 92.7%, while the specificity for the same type of bacteria was 90.4% (table 1).

The quantitative LAL test completed with the sequence of β-1,3-glucans blocking revealed the following limits (fig. 3):

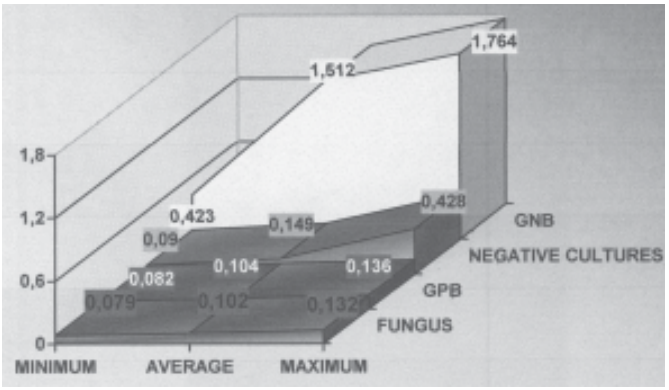


Fig. 3. Endotoxin levels (EU/mL) in the dialysate effluents at the time of diagnosis of peritonitis

-in peritonitis episodes with Gram-negative bacteria, the concentration of endotoxin ranged from 0.423 EU/mL to 1.764 EU/mL, with an average of 1.512 EU/mL;

-in peritonitis induced by Gram-positive bacteria, endotoxin concentration in the dialysis effluent varied between 0.082 EU/mL and 0.136 EU/mL, with an average of 0.104 EU/mL;

-in patients with sterile culture after 14 days of incubation, the concentration of endotoxins in the effluent varied between 0.090 and 0.428 EU/mL, with an average of 0.149 EU/mL;

-in fungal peritonitis (all Candida spp), the concentration of endotoxin ranged between 0.079 and 0.132 EU/mL, with an average of 0.102 EU/mL.

Statistical analysis of results from the quantitative LAL assay showed that the values of endotoxin concentration in effluent were significantly higher in Gram-negative peritonitis compared with the other types of peritonitis ($p=0.0001$). The ROC plot (with an area under curve of 99.93%, fig. 4) highlighted a sensitivity of 100% and a specificity of 95.89% – reached at endotoxin levels higher or equal to 0.423 EU/mL.

In peritonitis episodes with favorable response to treatment, repeating quantitative LAL test after curing the peritonitis episode, there were revealed the values from (fig. 5).

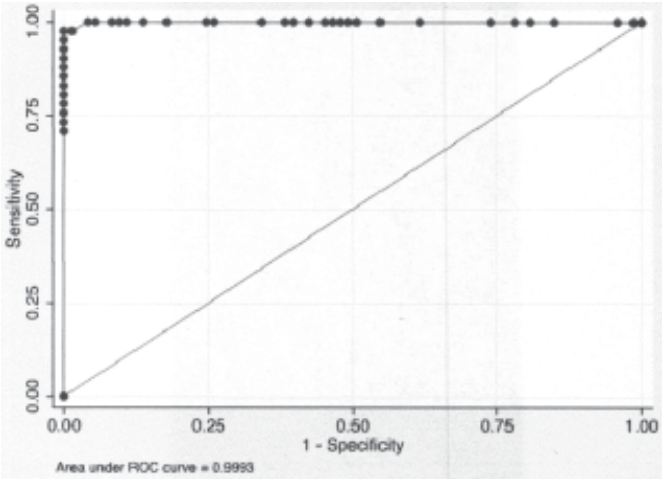


Fig. 4. The ROC plot (with an area under curve of 99.93%) showing a sensitivity of 100% that was reached at endotoxin levels greater than or equal to 0.423 EU/mL

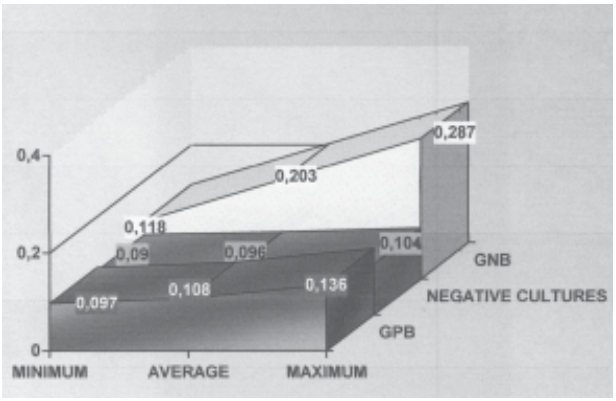


Fig. 5. The level of endotoxin (EU/mL) in dialysate effluent after the treatment of peritonitis

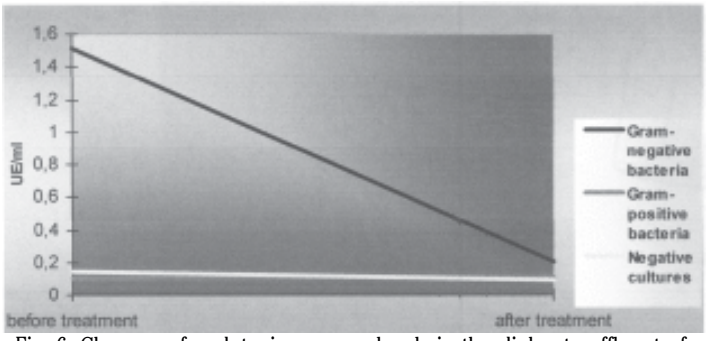


Fig. 6. Changes of endotoxin average levels in the dialysate effluent of peritonitis with favorable evolution under treatment

-in peritonitis with Gram-negative bacteria, the concentration of endotoxin ranged from 0.118 EU/mL and 0.287 EU/ml, with an average of 0.203 EU/mL;

-in peritonitis induced by Gram-positive bacteria, endotoxin concentration in the dialysate effluent varied between 0.097 EU/mL and 0.136 EU/mL, with an average of 0.108 EU/mL;

-in patients with negative cultures, the concentration of endotoxins in the effluent varied between 0.090 and 0.104 EU/mL, with an average of 0.096 EU/mL.

Statistical analysis of the variation of endotoxin levels in the effluent revealed that after the recovery of Gram negative peritonitis, the endotoxin concentration in the effluents decreased highly significant when compared to the other groups ($p = 0.0001$) – (fig. 6).

The study that we conducted reveals a high sensitivity and specificity of chromogenic quantitative LAL assay for the detection of peritoneal Gram-negative infections in CAPD patients. We also found that 0.423 UE/mL was the cutoff value above which the sensitivity of quantitative LAL test to detect Gram-negative infections was 97.6% and the specificity was 98.6%. These findings are in accordance with other studies that have used, with good results, LAL test for the detection of presence of Gram-negative bacteria in blood, cerebrospinal fluid, urine and peritoneal dialysis fluid [34-39,41-44]. Yet, LAL assays are not used in routine diagnosis of peritonitis in PD patients, various limitations being reported. Translocation of bowel bacteria in the peritoneum and blood may be a source of false positive reactions [45,46]. Vigorous handling of the effluent samples might lead to lysis of the bacteria with subsequent release of endotoxin and again with risk of false positive reactions [39]. Using LAL assays without blocking the reaction between LAL reagent and β-1,3-glucans may cause false positive reactions in case of fungal peritonitis [38]. Endotoxin shedding by Gram-negative bacteria differs between various strains of the same enterobacteria, thus false negative results of LAL test may arise [47,48]. LAL ability to react only with high molecular weight endotoxins is also criticized and is considered a possible cause of false negative results [49].

Regarding some of these concerns, in our research, centrifugation, stirring and thawing of effluent samples were avoided; the samples were collected by an instructed nephrologist trained to avoid any unwanted handling of the samples. Regarding the presence of endotoxins in the dialysate effluent in the absence of Gram negative bacteria, we found indeed a small, but detectable amount of endotoxin in patients with negative culture and with Gram-positive peritonitis (0.149 EU/mL and 0.108 EU/mL, respectively). But the difference between the amount present in Gram-negative peritonitis and the other types of peritonitis was notable and important. Furthermore, in patients with Gram-negative peritonitis, after cure of peritonitis, we revealed a significantly decreased of endotoxin levels, from an average of 1.512 to an average of 0.203 EU/mL, whereas in the other patients, endotoxin levels presented smaller changes (from an average of 0.104 EU/mL to 0.109 EU/mL in Gram-positive peritonitis, from an average of 0.149 EU/mL to 0.096 EU/mL in culture-negative peritonitis). Several researchers also reported presence of endotoxins or lipopolysaccharide-binding protein in effluents of peritonitis with Gram-positive bacteria [39, 50], but the significance of this findings remains to be elucidated.

Conclusions

Our study demonstrates that chromogenic LAL assay is a useful test in early diagnostic of peritonitis in CAPD patients.

Chromogenic screening LAL test had a specificity for detecting Gram-negative peritonitis of 92.7% and a specificity for the same type of bacteria of 90.4%. If only the screening LAL test is available, a positive result justifies selective antibiotic treatment with coverage on Gram-negative bacteria; in case of unfavorable evolution, the addition of antifungal therapy should be considered. A negative result cannot exclude a Gram-negative infection; therefore, broad spectrum antibiotic treatment should be administered until the results of the cultures are obtained.

Quantitative chromogenic LAL assay is the most precise tool for identify Gram-negative peritonitis and allows the choice of a more targeted treatment. False-positive reactions due to fungal infections can be prevented by blocking the reaction between LAL and b-1,3-glucans with the test β-G-blocker.

As in most devices, both tests (qualitative and quantitative) are available, errors in the choice of antibiotic therapy may be much reduced.

Therefore, routine LAL testing of dialysate effluents is recommended in CAPD patients admitted with peritonitis. Larger studies are needed to provide information about the exact cutoff value of endotoxin level above which the accuracy of the test for detecting Gram-negative induced peritonitis is maximum.

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Manuscript received: 22.12.2014