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THE LIMULUS AMOEBOCYTE LYSATE TEST: DETECTION OF ENDOTOXIN IN PLASMA OF SWINE AND CATTLE

I. IN VITRO INVESTIGATIONS

By

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MORTENSEN, K.and M. BINDER: *The Limulus Amoebocyte Lysate test: Detection of endotoxin in plasma of swine and cattle. I. In vitro investigations.* Acta vet. scand. 1985, *26*, 231—245. — Endotoxin added in vitro to plasma of swine and cattle was detected by the Limulus Amoebocyte Lysate (LAL) test in order to evaluate the possible utility of the test for detection of endotoxemia in the veterinary clinic and in research.

The inhibitory effect of plasma on the test was diminished in porcine plasma by dilution with saline, by heat-extraction, or by the pH-shift method. The last-mentioned method was not applicable to porcine plasma, whereas the heat-extraction method followed by serial 2-fold dilutions in water admitted the detection of endotoxin at low concentrations in porcine as well as in bovine plasma. The inhibition of the LAL test was more pronounced by bovine than by porcine plasma in that, when the same lysate sensitivity was used, the detection limit of endotoxin was highest in bovine plasma. The detection limit could be lowered by increasing the lysate sensitivity either by prolongation of the incubation or by the use of water instead of saline as diluent for plasma. However, an essential increase in lysate sensitivity demands extreme precautions, and a comparatively greater increase in the clinical applicability of the LAL test would be obtained if the inhibitory effect, that is present at low concentrations of endotoxin even in heat-extracted plasma, could be eliminated.

It is concluded, that to get full benefit of the LAL test for detection of endotoxemia in the veterinary clinic and in research, a functional LAL laboratory must be made available and new techniques for the elimination of plasma inhibitors at low concentrations of endotoxin must be developed.

plasma inhibitors; sensitivity; clinical laboratory methods; endotoxemia.

Limulus Amoebocyte Lysate (LAL) prepared from circulating blood cells of the horseshoe crab (Limulus polyphemus) is used for detection of minute amounts of endotoxin from gram-negative bacteria (*Levin & Bang* 1968), *Sullivan et al.* 1976, *Buchholz-Berchtold* 1977, *Jorgensen & Alexander* 1981). The principle of the test is a gelation of the lysate produced in the presence of endotoxin, or endotoxin-like activity, by activation of a pro-clotting enzyme and subsequent cleavage of a clottable protein (*Young et al.* 1972). The similarities of the gelation process to mammalian blood coagulation indicate, that an alternative pathway for the activation of the LAL pro-clotting enzyme might exist and conflicting reports have been published on the specificity of the test (*Reinhold & Fine* 1971, *Elin & Wolff* 1973, *Jorgensen & Smith* 1973, *Niwa et al.* 1974, *Wildfeuer et al.* 1974, *Yin* 1975, *Sullivan et al.* 1976, *Elin et al.* 1978, *Fink et al.* 1981).

The clotting may be inhibited by a high protein content in the test samples (*Nachum et al.* 1973) and detection of endotoxin in complex fluids such as blood, therefore, requires separation of the endotoxin from plasma inhibitors (*Levin et al.* 1970). Though the LAL test is relatively uncomplicated to perform when it is applied to simple fluids for merely qualitative purposes, difficulties may be encountered in quantitative analyses of complex fluids, and a high sensitivity, i.e., less than 1 ng endotoxin per ml water, will contribute to enhancing these difficulties.

In the present paper a description is given of low-cost procedures for detection of endotoxin in plasma of swine and cattle by the LAL test, and the utility of the test in the veterinary clinic and in research is evaluated by means of in vitro investigations.

MATERIALS AND METHODS

The LAL test should be performed in a separate room in the laboratory. Worktables and boxes with materials in use should be covered with sterile cloths and sterile gloves be worn constantly during the work. The water bath for incubation of the LAL test must be placed on a weighing table to prevent vibrations inhibiting the formation of a gel.

Preparation and wielding of utensiles

Disposable glass tubes for sample collection (16×100 mm) and assay (10×75 mm) are washed with a strong detergent and packed dustless by the manufacturer[1] under aseptical precautions. During each day's run used glass pipettes (25 µl and 100 µl ± 0.9 %, 900 µl ± 0.4 %)

[1] Bjørn Nielsen Hospitalsartikler A/S, Ny Solbjerg, Denmark.
[2] Deconex 11®, Borer Chemie AG, Solothurn, Switzerland.

are soaked in distilled water, tip upwards. At the end of the day the pipettes are rinsed for 15 min in hot running tap water and soaked in a 5 % solution of a strong detergent[2] in distilled water. After minimum 12 h and maximum 3 days the pipettes are again rinsed in hot running tap water for 15 min. The pipettes, thereafter handled separately, are further rinsed by aspiration of hot running tap water and distilled water, 10 times each. Aseptical, pyrogen-free water[3] is aspirated 10 times and absolute ethanol 5 times, and finally the pipettes are quickly dried with ether.

Aluminium boxes are soaked for 24 h in the detergent, rinsed thoroughly with tap water, distilled water, and pyrogen-free water and heated for 12 h at 250°C. Pieces of aluminium foil, glass tubes, and cleaned and dried pipettes are placed each within such processed aluminium boxes and heated at 250°C (*Tsuji & Harrison* 1978, *Tsuji & Lewis* 1978) for 12 h. Boxes with pyrogen-free materials are kept in plastic bags in a cupboard. Glass tubes in use are capped with pieces of heated foil and sealed with Parafilm.

Endotoxin

In the lack of reports on semiquantitative LAL analyses in connection with extensive veterinary experiments, a commercial endotoxin, E. coli O111:B4 LPS W.[4], was chosen for the present investigations in order to be able to compare our clinical experiments (*Binder & Mortensen* 1985) with those of *van Miert & Frens* (1968). The lyophilized endotoxin is reconstituted with pyrogen-free water to a concentration of 10^{-2} g/ml. The vial is vortexed for a minimum of 15 min or heated at 37°C for 30 min until the endotoxin solution is completely homogeneous. Serial 10-fold dilutions of the endotoxin solution are immediately prepared in pyrogen-free water. The error of dilution is minimized by sucking in and out 5 times at each step, vortexing vigorously for at least 30 sec, and subsequently changing the pipette. Endotoxin solutions containing 10^{-2} to 10^{-6} g/ml are stored vertically at —18°C. Solutions containing 10^{-7} to 10^{-9} g/ml are prepared every fortnight from 10^{-6} g/ml and stored at 2—8°C. Once a week 10^{-10} g/ml is prepared from 10^{-9} g/ml. All further dilutions are made daily. Saline[5] was used as diluent only when specified in the following description of Exp. 1.

Lysate

The lysate[6] is supplied lyophilized and is reconstituted with 5 ml of pyrogen-free water by gentle swirling of the vial, avoiding contact between the liquid and the rubber stopper. The reconstituted lysate is aseptically transferred to pyrogen-free glass tubes in aliquots of 0.3—1 ml. The glass tubes are carefully covered with aluminium foil and Parafilm and stored vertically at —18°C. The sensitivity of the lysate, i.e., the minimum concentration of endotoxin giving a positive test result, +, is 0.05—0.10 ng/ml water. Determination of the sensitivity is performed daily.

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[3] Sterile water for injection, Dispensary of the Royal Veterinary and Agricultural University, Denmark.

[4] Difco Laboratories, Detroit, Michigan, U.S.A.; control No. 673963.

[5] Isotonic sodium chloride for injection (9 mg/ml), DAK, Copenhagen, Denmark.

[6] Where nothing else is stated: Whittaker M.A. Bioproducts, Walkersville, Maryland, U.S.A.; lot No. 19682, 0.25 ng/ml FDA reference endotoxin.

Performance of the LAL test

25 µl of lysate is transferred to a maximum of 12 test tubes at a time, and 25 µl of the test solution is cautiously added to the lysate. Testing serial dilutions only one pipette is used for concentrations of 10^{-11} to 10^{-6} g/ml starting with the highest dilution of the sample. Each test tube is capped with aluminium foil and incubated in a water bath at a fixed temperature and period, cf. the experiments. All test samples are assayed in duplicate. Immediately after the incubation, the test tubes are cautiously removed from the water bath and turned upside down for reading. A firm, opaque gel adherent to the bottom of the tube is read as a positive reaction: +. A tube with contents remaining watery and unchanged in colour is read as negative: —. Intermediate reactions are graded as follows: increased turbidity and viscosity with a great number of floccules adherent to the sides of the inverted glass tube: (+); slightly increased turbidity and viscosity with only few floccules: (—).

Semiquantitation

The approximate concentration of endotoxin detected in a test sample is calculated by multiplying the sensitivity of the lysate by the end-point dilution factor, i.e., the factor designating the maximum dilution of the sample that still gives a positive result: +.

Collection of blood

The site of venipuncture is shaved, cleaned with ether and 70 % ethanol, and disinfected with a 5 % iodine solution. The iodine is afterwards removed with an Injection Swab[7]. After discarding the first 3—5 ml, 9 ml of blood is drawn with a sterile pyrogen-free disposable syringe[8] and needle[9], the syringe containing 1 ml of heparin[10]. Blood and heparin are mixed gently and transferred aseptically to pyrogen-free glass tubes and centrifuged for 10 min at 2,000 g. The plasma is removed with a pyrogen-free syringe and either stored at —18°C or processed immediately to eliminate the inhibitory effect and analysed.

Experiment 1: Detection of endotoxin in the plasma of swine

Pyrogen-free blood samples were collected from the jugular vein of healthy sows, and reference concentration of 10^4, 2.5×10^3, 625, 160, 78, 39, and 10 ng endotoxin per ml crude plasma were obtained by adding 100 µl of saline containing endotoxin in appropriate concentration to 900 µl of the pyrogen-free plasma. According to the method of *Reinhold & Fine* (1971), as applied to human plasma, the inhibitory effect of plasma was then eliminated by preparation of serial 2-fold dilutions in saline. The serially diluted plasma samples containing endotoxin were analysed, being incubated for 1 h at 37°C. Negative controls included saline and pyrogen-free plasma, the latter diluted serially 2-fold with saline. The concentration of endotoxin detected in the plasma samples was calculated by multiplying the sensitivity of the lysate[11] in saline by the end-point dilution factor, and expressed as a percentage of the original reference concentration.

In order to improve the assay, the inhibitory effect of plasma was eliminated by the pH-shift method introduced by *Reinhold & Fine* (1971) for human plasma. Reference concentrations of 500 ng and 8 ng endotoxin per ml crude plasma were obtained by adding 100 µl

[7] Injection Swab, Mölnlycke, Göteborg, Sweden.
[8] B-D®, Plastipak®, Becton Dickinson & Co., Ltd., Dublin, Ireland.
[9] Terumo, Mediplast AB, Mölndal, Sweden.
[10] Heparin® for Injection, 100 IU/ml, DAK, Copenhagen, Denmark.
[11] Lysate prepared by T. Mikkelsen, Denmark; lot. No. 801103.

of saline solutions containing, respectively, 5 μg and 0.08 μg endotoxin per ml to 900 μl plasma. The pH of the mixture was lowered and serial 2-fold dilutions in saline were analysed as above.

Finally, improvement of the LAL test was attempted by eliminating the inhibitory effect of plasma by the heat-extraction method of *Cooperstock et al.* (1975), as modified by *Berg et al.* (1979). The plasma was diluted 1:3 with pyrogen-free water, 1 part of plasma to 2 of water, vortexed and sealed carefully, and heated at 100°C for 20 min during which time the sample was vortexed twice. Test concentrations of 0.50, 0.25, and 0.10 ng endotoxin per ml heat-extracted plasma were obtained by adding 200 μl of endotoxin in appropriate concentration to 100 μl of the crude pyrogen-free plasma prior to heating. The theoretical concentration of endotoxin in the crude plasma sample was then 3 times as high as the test concentration. The heat-extracted plasma sample containing endotoxin was analysed along with its serial 2-fold dilutions. The incubation was 1 h at 37°C, negative controls included water and serial 2-fold dilutions of the heat-extracted pyrogen-free plasma. The concentration of endotoxin detected in the plasma samples was calculated by multiplying the sensitivity of the lysate in water by the end-point dilution factor and expressed as a percentage of the theoretical concentration in the crude plasma sample.

Experiment 2: Detection of endotoxin in the plasma of cattle

Preliminary observations indicated that the sensitivity of the lysate was not sufficient for detection of endotoxin in bovine plasma, when the plasma was subjected to heat-extraction and the assay was performed with 1 h of incubation at 37°C. The effect of continuing the incubation for 22 h at 20—23°C was investigated by determining the sensitivity of the lysate in water at the two different incubation procedures. The data were transformed to log 2, and the standard deviation (s), the coefficient of variation (C.V. %), and the geometric mean (G.M.) calculated (*Rastogi et al.* 1977). The geometric means were compared by a t-test.

The detection of endotoxin added to bovine plasma in small amounts was subsequently performed with both incubation procedures. Pyrogen-free blood samples were obtained from a healthy calf (No. 26) via an indwelling catheter[12] and the experiment was carried out like the last part of Exp. 1. Test concentrations and theoretical concentrations were, respectively, 3.30, 1.10, 0.56 ng per ml heat-extracted plasma and 10.00, 3.30, 1.68 ng per ml crude plasma.

RESULTS

Utensils prepared and wielded as described were found to be pyrogen-free when tested with a lysate with a sensitivity of 0.05 ng per ml water. A less cumbersome technique was functional only with a lysate sensitivity $\geq$ 1 ng per ml. The reconstituted lysate could be frozen, thawed and refrozen several times without loss of sensitivity, provided that the 12 test tubes for the assay were filled as soon as the lysate reached room temperature, and that cooling of the lysate or refreezing took place immediately after. When more than 12 test tubes were used at a time, heat radiation from the reading lamp and dust from different manipula-

[12] Braunüle®, B. Braun, Melsungen AG, West Germany.

tions might give rise to erroneous test results. The reading of the test was crucial, necessitating a standardized technique. Getting to know the reaction of one single lysate in various test solutions and at different concentrations of endotoxin contributed to increase uniformity. Plasma samples were especially difficult to evaluate at low dilutions because of the strong yellow colour and more or less pronounced cloudiness of both heat-extracted plasma and plasma treated by the pH-shift method. Furthermore, some plasma samples tended to coagulate on heating, and analysis of such samples could by performed only if a clear top layer was obtained after centrifugation at 1,800 g for 5 min.

With the surgical precautions described, contaminated blood samples were completely avoided, both on single sampling and on serial sampling via vein catheter. One "false positive" blood sample was obtained from a cow, which had recently experienced great stress.

The sensitivity of the LAL test decreased by factors of 3.7—9.5 when 0.15 mol/l saline was used as diluent instead of pyrogen-free water. The magnitude of this decrease seemed dependent on the lysate, in that the sensitivity of two different lysates[11], [13] was equal in water (0.21 and 0.16 ng/ml, $P >> 0.05$) while differing significantly in saline (2.00 and 0.59 ng/ml, $P < 0.01$).

Experiment 1: Detection of endotoxin in the plasma of swine

When the binding of endotoxin to inhibitors in plasma was dissociated by serial 2-fold dilutions in saline, endotoxin was easily detected by the LAL test at reference concentrations from 39 to 10^4 ng endotoxin per ml crude plasma, cf. Fig. 1. The percentage of endotoxin detected in the plasma samples was satisfactory, considering that the error of the stepwise quantitation achieved by serial 2-fold dilutions is ± one step; the detected low percentage of the 2 highest reference concentrations was presumably caused by the multiple dilutions. As illustrated in the left part of Fig. 1, the dilution necessary to separate endotoxin from the inhibitors of porcine plasma increased, when the concentration of in vitro added endotoxin was lowered. Consequently, 10 ng endotoxin per ml crude plasma could not be detected with certainty with a lysate having a sensitivity of 0.71 ng endotoxin per ml saline.

[13] Whittaker M. A. Bioproducts, Walkersville, Maryland, U.S.A.; lot No. 8581.

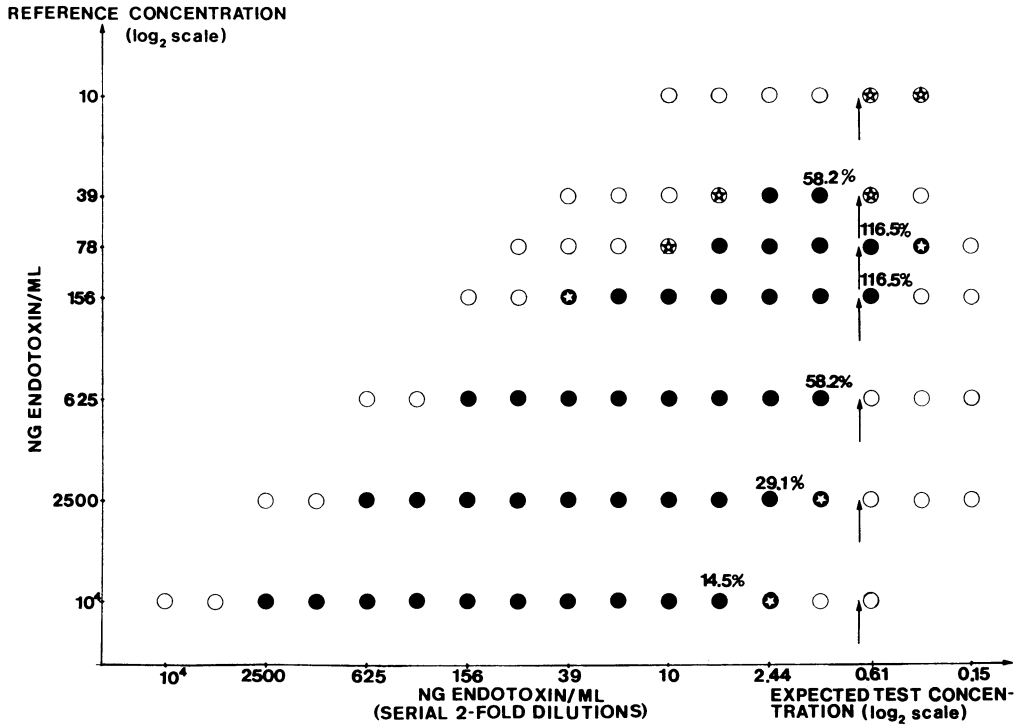

Figure 1. Detection of endotoxin in the plasma of swine by the LAL test following serial 2-fold dilutions in saline. The detected percentage of the original reference concentration is shown at the endpoint.

● = positive test result: +
✪ = intermediate test result: (+)
⊛ = intermediate test result: (—)
○ = negative test result: —
↑ = the sensitivity of the lysate, 0.71 ng/ml.

The pH-shift method of *Reinhold & Fine* (1971) for elimination of the inhibition lead to no improvement of the LAL test on porcine plasma, in that precipitation occurred and the inhibitory effect persisted, unlike what was the case with human plasma. 500 ng endotoxin per ml crude plasma could, therefore, only be detected when the pH-treated plasma was diluted 4-fold with saline as above, and 8 ng/ml could not be detected at all.

Heat-extraction and subsequent serial 2-fold dilution in water admitted the detection of endotoxin added in very small amounts to porcine plasma, cf. Table 1. With a lysate sensitivity of 0.08

Table 1. Detection of endotoxin in the plasma of swine by the LAL test following heat-extraction and serial 2-fold dilutions in water.

Theoretical concentration ng per ml crude plasma	Test concentration ng per ml extracted plasma	Sensitivity of the lysate ng per ml water	Test result of heat-extracted plasma	End-point dilution factor	Detected concentration	
					ng per ml crude plasma	% of theoretical conc.
0.30	0.10	0.08	—		0	0
0.75	0.25	0.08	(+)		<0.24[1]	<32
1.50	0.50	0.10	+	6	0.60	40
1.50	0.50	0.08	+	12	0.96	64

[1] Estimation of the concentration detected in samples with intermediate test result: below 3 times (i.e. the dilution factor in the heat extraction) the lysate sensitivity.

ng endotoxin per ml water the detection limit was between a theoretical plasma concentration of 0.75 ng and 1.50 ng endotoxin per ml. The detected percentage of the added endotoxin increased with rising plasma concentration and, at a given concentration, with improved sensitivity of the lysate.

Experiment 2: Detection of endotoxin in the plasma of cattle

Heat-extraction and subsequent serial 2-fold dilutions in water admitted the detection of endotoxin added in vitro to bovine plasma in small amounts, cf. Table 2. With a lysate sensitivity of 0.10 ng endotoxin per ml water the detection limit was be-

Table 2. Detection of endotoxin in the plasma of cattle by the LAL test following heat-extraction and serial 2-fold dilutions in water.

Theoretical concentration ng per ml crude plasma	Test concentration ng per ml extracted plasma	Sensitivity of the lysate ng per ml water[1]	Test result of heat-extracted plasma	End-point dilution factor	Detected concentration	
					ng per ml crude plasma	% of theoretical conc.
1.68	0.56	0.10	—		0	0
1.68	0.56	0.05	+	6	0.30	18
3.30	1.10	0.10	+	24	2.40	73
3.30	1.10	0.05	+	30	1.50	45
10.00	3.33	0.10	+	120	12.00	120[2]
10.00	3.33	0.05	+	240	12.00	120[2]

[1] The sensitivities of 0.10 ng/ml and 0.05 ng/ml correspond to incubation for 1 h at 37°C and 1 h at 37°C followed by 22 h at 20—23°C, respectively.

[2] Error probably caused by the stepwise quantitation.

Table 3. The effect of incubation procedures on the sensitivity of the lysate in water.

Incubation	n	$\log_2$ sensitivity Mean	s	C.V.%	Sensitivity ng/ml G.M.	Significance of difference
1 h at 37°C	14	0.571	0.756	132	0.15	P<<0.001
1 h at 37°C & 22 h at 20—23°C	23	—0.739	0.445	60	0.06	

tween a theoretical plasma concentration of 1.68 ng and 3.30 ng endotoxin per ml. Continuation of the usual incubation, 1 h at 37°C, for 22 h at 20—23°C lowered the detection limit to 1.68 ng endotoxin per ml crude plasma, or less, in consequence of an improved sensitivity of the lysate, cf. Tables 2 and 3. Like what was the case with porcine plasma, the detected percentage of the added endotoxin increased with rising plasma concentration.

DISCUSSION AND CONCLUSION

Endotoxin added in vitro to plasma of swine and cattle was detected by the LAL test provided that inhibition of the test was eliminated by heat-extraction or merely dilution of the plasma prior to analysis. The detected percentage of the added endotoxin depended on the method applied for separation of endotoxin from plasma inhibitors, the species of animal concerned, the sensitivity of the lysate, and the amount of endotoxin added. Furthermore, individual differences among the animals in the potency of plasma inhibitors may have had some influence, but the significance of this was not assessed.

Considering the differences in potency of endotoxins prepared from different sources by different methods (*Cooper et al.* 1971, *Elin & Wolff* 1973, *Jorgensen & Smith* 1973, *Niwa et al.* 1974, *Sullivan et al.* 1976, *Weary et al.* 1980) as well as the lysate-to-lysate, batch-to-batch, and laboratory-to-laboratory variability (*Rastogi et al.* 1977, *Rastogi et al.* 1979), a straight comparison between various records of semiquantitative LAL analyses is at the present of limited value, though the use of reference lysate and endotoxin and quality control procedues as described in the two last-mentioned papers should allow direct comparison in the future.

Injection of endotoxin in vivo followed by detection of endo-

toxin in plasma according to the methods described in this paper, revealed that symptoms of endotoxemia were evident in swine and cattle at or below calculated plasma concentrations of 6 ng endotoxin per ml (*Binder & Mortensen* 1985). Heat-extraction of plasma alone admitted the detection of endotoxin at suitable plasma concentrations and, therefore, was found to be superior to merely dilution for the separation of endotoxin from plasma inhibitors. *Smith et al.* (1982) compared 3 different methods for elimination of equine and bovine plasma inhibitors, and found the heat-extraction method superior to the chloroform-extraction method (*Levin et al.* 1970) and the PSI Bead Assay (*Harris & Feinstein* 1977) in both species. Unfortunately, the weight of the work is weakened by some false positive results. The pH-shift method introduced by *Reinhold & Fine* (1971) for use with human plasma was found, in the present study, not to be applicable to Porcine plasma.

In bovine plasma the detection limit of endotoxin is higher than in porcine plasma and the inhibitory effect, therefore, must be more pronounced. Likewise, comparing equine and bovine blood, *Berg et al.* (1979) and *Smith et al.* (1982) found the greatest inhibition of the LAL test in bovine blood. By the heat-extraction method *Berg et al.* (1979) were able to detect endotoxin added to whole blood of both swine and cattle to a final concentration of 1 ng per ml blood, when the lysate sensitivity was 0.01 ng endotoxin per ml water, but no estimate was made of the percentage detected or of possible species-associated differences.

The superiority of the heat-extraction method for elimination of the inhibitory effect of plasma on the LAL test may be caused by several factors: Instead of saline used as diluent for plasma, when the inhibition is eliminated by merely dilution, the diluent of the heat-extraction method is water, which results in a lysate-dependent increase in sensitivity. This inhibitory effect of saline, even in physiological concentrations, is consistent with the findings of other investigators (*Schleef et al.* 1979, *Weary et al.* 1980).

Another advantage of the heat-extraction is the heating itself, as unheated serum or plasma possesses a very marked endotoxin detoxifying capacity (*Skarnes et al.* 1958). By the LAL test *Webster* (1980) proved, that in fresh human serum, taken from healthy volunteers, 600 ng endotoxin per ml per h was inactivated during the first 3 h of incubation at 37°C. After 3 h, detoxifica-

tion will proceed more slowly, but even at 5°C it will be substantial after 24 h. Similar results have been obtained with platelet-rich plasma (*Thomas et al.* 1981). *Skarnes & Rosen* (1971) believe that the detoxification is a two-stage-reaction, the first part, which is inhibited by Ca-ions, being an anion-dependent binding and degradation of endotoxin by a heat-stable arylesterase associated with *H*igh *D*ensity *L*ipoprotein (*Freudenberg et al.* 1980), the second part being an enzymatic detoxification by a heat-labile α_1-globulin esterase. Heating of plasma, therefore, prevents an irreversible loss of endotoxin, while dilution of plasma dissociates a reversible binding of endotoxin to plasma proteins (*Levin et al.* 1970).

From the above-mentioned it is obvious, that prior to the heating all handling of blood samples containing endotoxin must be very fast. If, e.g., whole blood and endotoxin is incubated at 37°C, then endotoxin will associate with lymphocytes, erythrocytes, and platelets, or be taken up by leukocytes and monocytes, or be detoxified by plasma esterases. By the LAL test a very low percentage of the added endotoxin will be detected, as also experienced by *Maxie & Valli* (1974), who incubated bovine whole blood and endotoxin for 3 h at 37°C.

From the clinical experiments previously mentioned (*Binder & Mortensen* 1985) it may also be seen, that in spite of clinical symptoms of endotoxemia the detoxification of endotoxin in vivo to levels below the test sensitivity is very rapid too, and consequently there is a need to improve the technique. Some improvement of the test was obtained in the present study by simple means, i.e., the previous mentioned use of water instead of saline as diluent for plasma and the prolangation of the incubation procedure. The positive influence of prolonged incubation was to be expected, since the rate of gelation of the lysate is related to the concentration of endotoxin (*Levin & Bang* 1968), but for the clinical use of the LAL test incubation for 24 h is clearly a disadvantage. An essential improvement of the sensitivity requires extreme precautions that are not easily complied with, and though the detection limit of endotoxin in plasma may be lowered, the basic problem is that the detection limit is not equal or nearly equal to the sensitivity of the lysate in water; an inhibitory effect of plasma thus is still present at low plasma concentrations of endotoxin, in spite of the heat-extraction. Comparing the lowest test concentrations, the test results, and the sensitivity of

the lysate in Tables 1 and 2, the persistent inhibition is evident. Some endotoxin may not be detected by the LAL test if not dissociated from the binding to plasma proteins or if it is detoxificated by plasma esterases prior to the heat-extraction; the detoxification is, however, the least probable cause, as all of the added endotoxin is detectable at the highest concentrations. The finding that, in spite of the heat-extraction, the inhibition of the LAL test increases when the plasma concentration of endotoxin is lowered, is consistent with the feature of the inhibition when porcine plasma is not heat-extracted but only diluted with saline. This feature was also exhibited by human plasma (*Reinhold & Fine* 1971), only the inhibition was greater than that of porcine plasma. In order to increase the clinical applicability of the LAL test, an improved technique for the elimination of plasma inhibitors at low endotoxin concentrations must be developed. Actually, *Hollander & Harding* (1976) succeeded in detecting 75—100 % of 0.5—500 pg endotoxin added in vitro per ml murine plasma, by using a chromatographic method for separation of endotoxin from inhibitors of platelet-rich plasma, a method that might qualify for more widespread use.

It is concluded that the LAL test may still prove to be valuable in the veterinary clinic for the detection of endotoxemia and in research. However, the high sensitivity needed for detection of endotoxemia and the multiple sources of error of the LAL analysis on blood imply that a functional LAL laboratory must be made available to the investigator. Furthermore, improved methods must first be developed for the elimination of the inhibitory effect of animal plasma at low concentrations of endotoxin, since even the heat-extraction method was not adequate at the lowest concentrations, where essential inhibition persisted.

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SAMMENDRAG

Limulus Amøbocyt Lysat testen: Påvisning af endotoksin i svine- og kvægplasma. I. In vitro undersøgelser.

Med henblik på at vurdere den mulige anvendelse af LAL testen til påvisning af endotoksinæmi i den veterinære klinik og i forskningsøjemed blev in vitro tilsat endotoksin søgt påvist i plasma fra svin og kvæg.

Plasmaets evne til at inhibere testen blev formindsket i svineplasma ved fortynding med saltvand, ved varme-ekstraktion eller ved pH-skifte. Den sidstnævnte metode havde ingen effekt på svineplasma, hvorimod varme-ekstraktion efterfulgt af en 2-folds fortyndingsrække i vand åbnede mulighed for påvisning af endotoksin i lave koncentrationer i både svine- og kvægplasma. Kvægplasmaets inhibition af LAL testen var mere udtalt end svineplasmaets, da påvisningsgrænsen for endotoksin var højest i kvægplasma, når lysat med samme følsomhed blev anvendt. Påvisningsgrænsen kunne nedsættes ved at øge lysatets følsomhed enten ved forlængelse af inkubationstiden eller ved at bruge vand til fortynding af plasma i stedet for saltvand. En væsentlig øgning af lysatets følsomhed kræver imidlertid, at der tages ekstreme forsigtighedsforanstaltninger, og en forholdsvis større forbedring af LAL testens kliniske anvendelighed ville opnås, hvis man helt kunne eliminere den inhibition, der til trods for varme-ekstraktion af plasmaet er til stede ved lave koncentrationer af endotoksin.

Det konkluderes, at for at få fuldt udbytte af LAL testen til påvisning af endotoksinæmi i den veterinære klinik og i forskningsøjemed må et laboratorium med rutine i anvendelsen af testen være tilgængeligt, og samtidig må nye metoder til elimination af plasmaets inhibition af testen ved lave endotoksinkoncentrationer udvikles og afprøves.

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