Antonie van Leeuwenhoek **66:** 333–336, 1994.

Detection of *Bacteroides fragilis* endotoxin in amniotic fluid by counterimmunoelectrophoresis

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Accepted 2 May 1994

Key words: B. fragilis, endotoxin, counterimmunoelectrophoresis, ultrafiltration, amniotic fluid

Abstract

The ability of counter immunoelectrophoresis (CIE) to detect *Bacteroides fragilis* endotoxin in amniotic fluid in small concentrations was evaluated. A method was developed which, in combination with ultrafiltration, permits detection of *B. fragilis* endotoxin in amniotic fluid in a concentration of 40 ng/ml or more. The sensitivity threshold was reduced to 2 ng/ml by using a highly reactive IgG-fraction isolated from rabbit anti-*B. fragilis* IPL E 323 antiserum.

Introduction

Counterimmunoelectrophoresis (CIE) has been successfully employed for the detection of polysaccharide antigens isolated from various species of the genus *Streptococcus, Pneumococcus, Haemophilus* and *Neisseria* in serum (Shackelford et al. 1974; Ingram et al. 1972; Granoff et al. 1977; Shetty et al. 1985), liquor cerebrospinalis (Shackelford et al. 1974; Ingram et al. 1972; Granoff et al. 1977), urine (Shackelford et al. 1974), sputum (El-Refaie & Dulake 1975; Ericsson et al. 1986) and tears (Powell et al. 1988). *Streptococcus B* polysaccharide has also been detected in amniotic fluid (Jacobs et al. 1981). The specific detection of lipopolysaccharides (LPS), the endotoxins of gram negative bacteria, by CIE in amniotic fluid has not yet been described.

During our study of the maternal and fetal immuneresponse to *B. fragilis* endotoxin in pregnant guinea pigs (Beckmann et al. 1991) it became necessary to assess in amniotic fluid the presence of *B. fragilis* endotoxin in low concentrations. This present study describes a modified system of CIE for the specific detection of *B. fragilis* endotoxin in small quantities in amniotic fluid.

Material and methods

B. fragilis *endotoxin*

Bacteria (*B. fragilis* IPL E 323) were extracted with phenol/water and the lipopolysaccharide isolated as described by Beckmann et al. (1989).

Antisera

Rabbit antisera against *B. fragilis* IPL E 323 bacteria were prepared as described by Meisel-Mikołajczyk et al. (1981). The antisera $E_2 8$ and $E_2 9$ were from different rabbits immunized with the same bacterial suspension.

Isolation of IgG from B. fragilis *antiserum*

According to the method of McKinney & Parkinson (1987) rabbit anti *B. fragilis* antiserum was precipitated with n-caprylic acid, cooled to 4° C and fractionated with ammonium sulfate (45% saturation). After stirring for 30 min the precipitated IgG was collected by centrifugation at 2000 g for 20 min. The precipitate was dissolved in a small volume of phosphate buffered saline (PBS) and dialysed at 4° C over night against PBS. After heating for 20 min at 55° C the protein

content of the solution was determined by absorption spectrometry at 260 nm.

IgG_1 was isolated from antiserum $E_2(83)$, IgG_2 from antiserum *B. fragilis* $E_2(8)$. The sera were obtained by immunization of rabbits with different preparations of bacterial suspensions.

Amniotic fluid

Amniotic fluid from healthy pregnant guinea pigs was isolated on day 61 of the gestation, centrifuged at 1500 rpm, and the clear supernatant stored at - 20° C until assay.

Ultrafilters

Ultrafilters UFC3 LCC00 (Molecular weight (MW) limit MW < 5000 D) and UFC3 LGCOO (MW limit MW < 10000 D) were from Millipore B.V., Etten-Leur, The Netherlands.

Chemicals

All chemicals were from Sigma, St. Louis, U.S.A. and Merck, Darmstadt, Germany.

Counterimmunoelectrophoresis (CIE)

CIE was performed on microscopic slides covered with 2 ml of agarose gel (1% agarose in Laurell buffer (pH 8.6). After electrophoresis the slides were incubated over night in a humid chamber at 4° C in PBS and stained according to the method of Weeke (Weeke 1973) with Coomassie Brilliant Blue G 225. All results were assessed blinded, by an independent investigator.

Grabar-immunoelectrophoresis

The one-dimensional immunoelectrophoresis on microscopic slides was performed as described by Beckmann et al. (1985).

Ultrafiltration of amniotic fluid

LPS dissolved in amniotic fluid was kept for 3 min at 100° C and 400 µl of the solution were applied to an ultrafilter and centrifuged for 60 min at 2000 g and 20° C. New solution was added, until the original solution remaining in the filter was concentrated tenfold. The volumes of remaining and filtered fluids were measured and kept at - 20° C until further testing.

Table 1. Minimal detectable concentration of *B. fragilis* LPS in CIE depending on antibody source.

Antibody source	Number of tests	Detectable concentrations LPS (µg/ml)
Antiserum E_2[8]	15	0.31–0.63
Antiserum E_2[9]	3	0.31–0.63
IgG-fraction (1)	3	0.02–0.03
IgG-fraction (2)	3	0.75–1.56

Results

The optimum sensitivity threshold for the detection of *B. fragilis* LPS with CIE was determined by varying the conditions (quantity of antibody, distance between antigen and antibody reservoirs, field strength and duration of electrophoresis). The final procedure was as follows: On agarose-covered microscopic slides two rows of holes (diameter 3.5 mm) with a distance of 3 mm between rows were applied. *B. fragilis* LPS, dissolved in PBS (pH 7.4), was diluted 1:2 in amniotic fluid, and 6 µl of each dilution were placed into the holes directed towards the cathode; 8 µl antiserum were put into the holes directed towards the anode. Controls for amniotic fluid and PBS were included. After electrophoresis for 45 min at a field strength of 3.2 V/cm and 20° C, the immunoprecipitates were processed as described above. Under these conditions 310–630 ng of *B. fragilis* LPS could be detected.

The detection limit depends on the concentration of antibodies within the antibody source. Table 1 shows the detectable LPS-concentrations after application of different sources of antibodies in the CIE test.

The ultrafiltration of amniotic fluid, containing *B. fragilis* IPL E 323 LPS, was performed with two types of ultrafilters (5000 and 10000 D), permitting the passage of substances with a molecular weight < 5000 D or < 10000 D. The permeability of both filters for the LPS of *B. fragilis* was investigated by Grabar immunoelectrophoresis and CIE of residues and filtrates after ultrafiltration of 500 µg LPS dissolved in 1 ml amniotic fluid. Figure 1 shows the results of Grabar immunoelec-

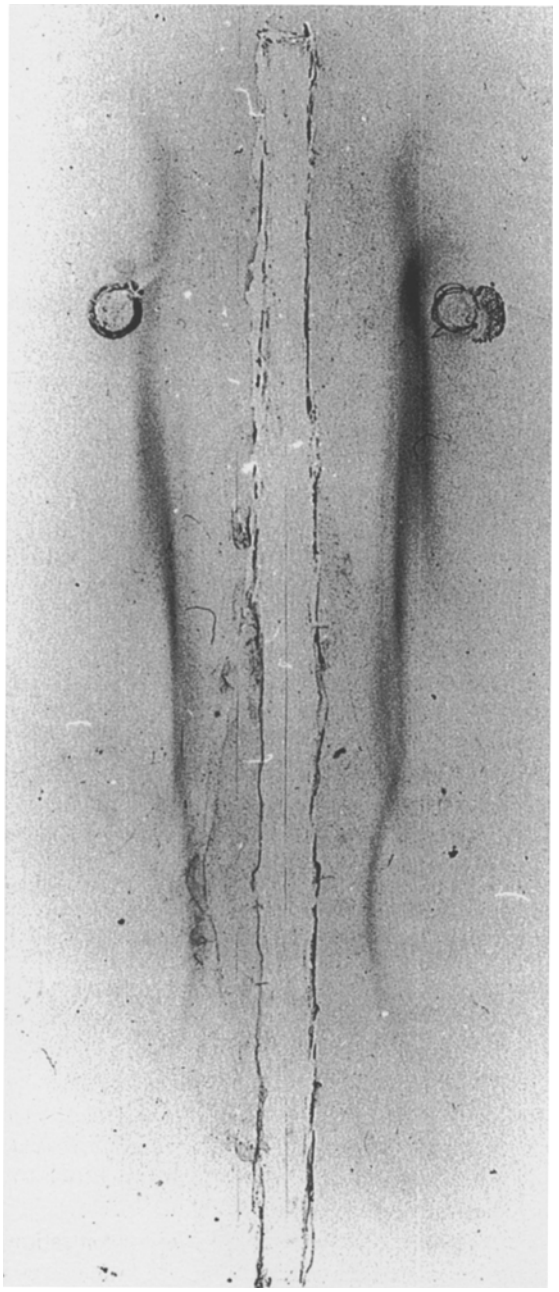

Fig. 1. Immunoelectrophoresis of *B. fragilis* lipopolysaccharide (LPS) before and after ultrafiltration. Left: LPS before ultrafiltration, right: LPS in the residue after ultrafiltration through a 5000 D filter, middle: *B. fragilis* antiserum.

trophoresis of the residue after ultrafiltration through the 5000 D filter, and of the original LPS-solution.

The residue still contains all components of the heterogenous LPS-fraction, and the filtrate did not react with a visible precipitate. CIE with this filtrate was also negative, in contrast to the reaction of the filtrate obtained through the 10000 D filter, in which a small quantity of LPS could be detected. Filtration of an LPS-solution in amniotic fluid through a 5000 D-filter gave quantitative results as shown for one example in Table 2.

The LPS-concentration in the residue was calculated from the final titer in a CIE-test, compared with the titer of the original, unfiltered solution.

The sensitivity threshold of CIE, after tenfold concentration of the antigen solution by ultrafiltration, was determined as 40 ng LPS/ml amniotic fluid (antibody source rabbit-anti *B. fragilis* antiserum) and 2 ng LPS/ml (antibody source IgG).

Discussion

Detection of endotoxins in amniotic fluid by CIE has not yet been described. In connection with intrauterine infections, endotoxins were mainly detected by the limulus amebocyte lysate assay, which is very sensitive, but does not permit identification of the endotoxin. The sensitivity reached by combining ultrafiltration of lipopolysaccharides with CIE is of the same order of magnitude as that described for the detection of capsular polysaccharides by CIE in other body fluids, but lower than the sensitivity of the limulus assay.

For the ultrafiltration of a lipopolysaccharide the molecular weight of the concentrated substance must be known and the pore size of the filter membrane should be carefully adjusted. Although the molecular weight of *B. fragilis* LPS of 12000 D (Kasper & Seiler 1975) was above the indicated limit of the 10000 D filter, antigenic components were present in the filtrate, which made the use of a filter with a smaller pore size necessary.

The CIE results obtained with different batches of anti *B. fragilis* antiserum and IgG indicate that the sensitivity depends heavily on the antibody source. With a highly reactive IgG-preparation, the sensitivity was almost tenfold increased and, in combination with ultrafiltration, reached a detection limit of 2 ng/ml.

The method as described in this report is useful for the specific detection of small quantities of endotoxins in amniotic fluid.

Table 2. Concentration of *B. fragilis* LPS in amniotic fluid by ultrafiltration through a 5000D-filter.

Applied	in	Filtrate	Residue	Theoretic concentration in residue	Detected by CIE	
μg LPS	μl	μl	μl	μg/ml	μg/ml	(%)
1.25	1000	920	60	21	20	95

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