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Diagnosis of Endotoxemia with Gram-Negative Bacteremia Is Bacterial Species Dependent: a Meta-Analysis of Clinical Studies[▽]

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Endotoxemia is undetectable for up to 60% of cases of bacteremia caused by gram-negative (GN) species, a discordance attributed to the limitations of the *Limulus* assay for endotoxemia. The lipid A structure of the endotoxin molecule is critical for the sensing of GN bacteria by the host immune system although not so for sensing by the *Limulus* assay. The lipid A structure of commensal *Enterobacteriaceae* is hexa-acyl, whereas non-*Enterobacteriaceae* have a broader range of structures. By using a previously published classification of lipid A structures (R. S. Munford, Infect. Immun. 76:454–465, 2008), the association of endotoxemia with bacteremia caused by GN organisms is reexamined for 580 GN bacteremic patients from 46 studies. Endotoxemia was less commonly detected for cases of bacteremia caused by *Salmonella enterica* serovar Typhi (four studies; 15 of 55 cases of bacteremia [27%]) than for cases of bacteremia caused by *Neisseria meningitidis* (five studies; 69 of 84 cases [82%]) and *Pseudomonas pseudomallei* (one study; 38 of 41 cases [93%]) among studies restricted to those with specified cases of bacteremia caused by GN organisms. Among 23 unrestricted studies, endotoxemia was less commonly detected for cases of bacteremia with a commensal member of the *Enterobacteriaceae* (104 of 240 cases [43%]) than with non-*Enterobacteriaceae* (59 of 100 cases [59%]) (summary odds ratio, 0.53 [90% confidence interval, 0.33 to 0.85]). This finding is consistent across all the unrestricted studies, even including studies with seemingly contrary results for endotoxemia diagnosis among cases of bacteremia caused by GN bacteria overall. Surprisingly, with bacteremia caused by commensal *Enterobacteriaceae*, the diagnosis of endotoxemia appears to be unrelated to the *Limulus* assay sensitivity. Across these 45 studies, the association of endotoxemia with GN bacteremia is variable but consistent for different types of GN bacteremia.

There are key structural differences between the lipid A components of the endotoxin molecule (lipopolysaccharide) of different gram-negative (GN) bacteria. Members of the *Enterobacteriaceae* characteristically have a lipid A structure with a hexa-acyl structure, whereas other lipid A structures are present for non-*Enterobacteriaceae* (45). These differences in lipid A structure are now known to be critically important for the recognition of GN bacteremia by the host immune system (45) by the MD-2–Toll-like receptor 4 receptor but not for sensing by the clotting proteins of the blood cells of the *Limulus polyphemus* horseshoe crab, from which the *Limulus* amebocyte lysate assay is derived (56). If the recognition of hexa-acyl lipid A by the host immune system has a role in the pathogenesis of bacteremia, it might be expected that the proportion with detectable endotoxemia among patients with GN bacteremia might depend on the lipid A structure of the isolate.

Attempts to define the concordance between GN bacteremia and endotoxemia in patients with sepsis have been elusive. Indeed, two of those studies (15, 54), with over a hundred patients each, concluded that there is no concordance. The purpose of this review is to attempt to reconcile the disparate findings from studies of clinically detected sepsis by examining the proportion with endotoxemia detected by using the *Limulus* assay for patients with different types of GN bacteremia for which the lipid A structures are known. Of particular interest is the proportion with endotoxemia among cases of bacteremia caused by commensal *Enterobacteriaceae* versus non-*Enterobacteriaceae*. The statistical techniques of meta-analysis are used to derive study-specific and summary estimates of these proportions expressed as an OR and more so to obtain estimates of the consistency in these ORs across the panel of studies (24).

MATERIALS AND METHODS

Data sources. A comprehensive search of the literature from 1966 to April 2009 was performed by using a search strategy detailed previously (27, 29), and a call for data was issued (30). Clarifications of published data were sought from the original authors. This search was in addition to publications obtained from a library of several hundred publications related to clinical aspects of endotoxemia that I accumulated from repeatedly searching the literature over two decades (29, 33).

Study selection and classification of bacteria. The following inclusion criteria were used: (i) comparison of the *Limulus* assay with blood cultures from patients with suspected GN bacteremia, (ii) the sensitivity of the *Limulus* assay to an internal endotoxin standard stated, and (iii) a minimum of two patients with GN bacteremia.

The studies were classified regarding whether they were restricted to examining one of four specific GN bacteremias (*Salmonella enterica* serovar Typhi, *Neisseria meningitidis*, *Yersinia pestis*, and *Burkholderia pseudomallei*) for which characteristic clinical features facilitate the diagnosis (restricted studies) versus studies that were unrestricted (unrestricted studies). For the unrestricted studies, the bacteria were classified into two groups according to the lipid A structure reported previously (see Table 1 of reference 45) into those known to have a hexa-acyl lipid A structure and those known to have a non-hexa-acyl lipid A structure. The group of bacterial isolates known to have a hexa-acyl lipid A structure is labeled commensal *Enterobacteriaceae* for the purposes of this analysis, as in the clinical setting, this group consists predominantly of commensal *Enterobacteriaceae* (*Escherichia coli*, *Klebsiella pneumoniae*, *Serratia marcescens*, *Proteus*

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TABLE 1. Detection of endotoxemia in association with GN bacteremia[a]

Authors, yr (reference)	*Limulus* assay sensitivity limit (ng/ml)	No. (%) of patients with GN bacteremia caused by:			
		Commensal *Enterobacteriaceae*[b]		Non-*Enterobacteriaceae*[c]	
		Limulus positive	*Limulus* negative	*Limulus* positive	*Limulus* negative
Assay band A[d]					
van Dissel et al., 1993 (58)	0.001	3	0	ND	ND
Goldie et al., 1995 (22)[e]	0.002	2	1	3	1
Yoshida et al., 1993 (61)[e]	0.003	3	3	9	3
Ahmed et al., 2004 (2)	0.004	5	1	3	0
Massignon et al., 1996 (42)[e]	0.005[f]	13	2	4	2
Dofferhoff et al., 1992 (14)	0.005	2	2	2	0
Guidet et al., 1994 (23)[e]	0.005	13	6	6	0
Ketchum et al., 1997 (37)[e]	0.005	8	32	2	6
van Deventer et al., 1988 (57)	0.005	14	4	1	0
Subtotal for assay band A		63 (55)[f]	51 (45)[f]	30 (71)[g]	12 (29)[g]
Assay band B[d]					
Danner et al., 1991 (11)[e]	0.01	3	4	2	0
McCartney et al., 1987 (43)	0.01	0	1	1	0
Shenep et al., 1988 (52)	0.025	3	1	1	0
Giamarellos-Bourboulis et al., 1999 (21)[e]	0.01	9	1	ND	ND
Hass et al., 1986 (26)	0.01	ND	ND	2	0
Suyasa et al., 1995 (55)	0.01	ND	ND	4	0
Hynninen et al., 1995 (34)	0.013	2	18	0	3
Bion et al., 1994 (3)	0.02	ND	ND	1	1
Exley et al., 1992 (16)	0.025	2	0	ND	ND
Subtotal for assay band B		19 (43)[f]	25 (57)[f]	11 (73)[g]	4 (27)[g]
Assay band C[d]					
Prins et al., 1995 (49)[e]	0.04	4	5	1	0
Fossard and Kakkar, 1974 (19)	0.1	1	0	1	0
Pearson et al., 1985 (47)	0.1	6	1	ND	ND
Fink and Grunert, 1984 (18)	0.1[f]	1	0	2	0
Scheifele et al., 1985 (51)	0.2	2	1	ND	ND
Stumacher et al., 1973 (54)	0.5	14	26	5	8
Oberle et al., 1974 (46)	0.5	1	0	2	0
van Wieringen et al., 1976 (59)	0.5	3	0	ND	ND
Subtotal for assay band C		32 (49)[f]	33 (51)[f]	11 (58)[g]	8 (42)[g]
Assay band D[d]					
Cooperstock and Riegle, 1985 (10)	1	3	0	3	1
Feldman and Pearson, 1974 (17)	1	0	12	2	12
Jirillo et al., 1975 (35)	1	1	1	ND	ND
Lau et al., 1996 (38)	1[f]	8	3	ND	ND
Clumeck et al., 1977 (9)	3	5	3	2	0
Kelsey et al., 1982 (36)	5	0	1	1	0
Levin et al., 1972 (39)	5	10	9	6	1
Martinez et al., 1973 (41)	5	1	6	0	3
Subtotal for assay band D		28 (44)[g]	35 (56)[g]	14 (45)[h]	17 (55)[h]
Total excluding studies with ND		108 (44)	137 (56)	59 (60)	40 (40)
Total all studies		142 (49)	144 (51)	66 (62)	41 (38)

[a] The following bacteria causing bacteremia (with numbers of *Limulus* amebocyte lysate-positive patients and the total numbers of patients) from 17 studies (2, 9, 10, 11, 17, 22, 23, 34, 37, 39, 41, 52, 54, 57, 58, 59, 61) are not included in this analysis: *N. meningitides* (six of six), *Salmonella* species (seven of nine), *Enterobacter* species (13 of 33), and *Haemophilus influenzae* (eight of nine). ND, no data.

[b] Commensal *Enterobacteriaceae* as defined in Materials and Methods.

[c] Non-*Enterobacteriaceae* include 74 cases of bacteremia with *Pseudomonas* species, 6 cases of bacteremia with *Acinetobacter* species, and 20 cases of bacteremia with anaerobic bacteria (*Fusobacterium* species and *Bacteroides* species).

[d] The assay bands and assay sensitivity limits of the included studies are as defined in Materials and Methods.

[e] Data were clarified by personal communication from study authors.

[f] The *Limulus* assay sensitivity limit in these studies was expressed in endotoxin units per ml, and a conversion equivalence of 1 endotoxin unit/ml to 100 pg/ml of endotoxin has been used here.

[g] Test for equality of the proportion of patients with endotoxemia among patients with bacteremia and a commensal *Enterobacteriaceae* isolate detected among subtotals of bands of studies (chi-squared value, 2.27; $P = 0.52$; 3 df).

[h] Test for equality of the proportion with endotoxemia among patients with bacteremia and an non-*Enterobacteriaceae* isolate detected among subtotals of bands of studies (chi-squared value, 5.7; $P = 0.13$; 3 df) with a test for trend (chi-squared value, 4.9; $P = 0.03$; 1 df).

TABLE 2. Detection of endotoxemia in association with bacteremia among studies of four specified GN infections (restricted studies)

Organism and authors, yr (reference)	Assay sensitivity limit (ng/ml)	No. (%) of patients	
		Limulus positive	*Limulus* negative
Neisseria meningitidis			
Bjorvatn et al., 1984 (4)	0.005	4	0
Brandtzaeg et al., 1989 (5)	0.005	24	11
Gardlund et al., 1995 (20)	0.01	9	1
Prins et al., 1998 (48)	0.04	30	1
Harthug et al., 1983 (25)	0.5	2	2
Subtotal for *N. meningitidis*		69 (82)	15 (18)
Yersinia pestis			
Butler et al., 1973 (7)	0.5	2	0
Butler et al., 1976 (8)	5	3	2
Subtotal for *Y. pestis*		5 (71)	2 (29)
Burkholderia pseudomallei			
Simpson et al., 2000 (53)	0.002	38 (93)	3 (7)
Salmonella serovar Typhi			
McGladdery et al., 1993 (44)	0.04	0	16
Adinolfi et al., 1987 (1)	0.3	7	7
Butler et al., 1978 (6)	1	0	13
Magliulo et al., 1976 (40)	1	8	4
Subtotal for *S. Typhi*		15 (27)	40 (63)

mirabilis, and *Providencia rettgeri*). The group of bacterial isolates known to have a non-hexa-acyl lipid A is labeled as non-*Enterobacteriaceae* for the purposes of this analysis. Among the unrestricted studies, there were small numbers of the four specified GN bacteria, members of the *Enterobacter* species (which can have either a hexa-acyl [*Enterobacter cloacae*] or non-hexa-acyl [*Enterobacter agglomerans*] lipid A structure) and *Haemophilus influenzae* (hexa-acyl lipid A structure), and these were all analyzed separately.

Data extraction and analysis: calculation of an OR. The endotoxemia diagnosis data for all studies were extracted for all those with GN bacteremia on a per-patient basis. For those unrestricted studies with data available for both commensal *Enterobacteriaceae* and non-*Enterobacteriaceae*, the data were extracted into a 2-by-2 contingency table format based on the diagnosis of endotoxemia (*Limulus* positive) versus no diagnosis (*Limulus* negative) and the type of GN bacteremia (commensal *Enterobacteriaceae* versus non-*Enterobacteriaceae*) to enable the calculation of a study-specific OR and 90% confidence interval (CI). The summary OR and 90% CI were derived by using the Metan command (24) in STATA (release 10.0; STATA Corp., College Station, TX).

Assay sensitivity. The sensitivity of the *Limulus* assay used in each study is the sensitivity to the internal endotoxin standard stated in each study. The unrestricted studies were stratified into four bands of assay sensitivity as follows: <0.01 ng/ml (assay band A), ≥ 0.01 to 0.03 ng/ml (assay band B), 0.033 to 0.9 ng/ml (assay band C), and ≥ 1 ng/ml (assay band D). A nonparametric test for trend was analyzed by using the subtotals derived for each band. The trend in the proportion with detectable endotoxemia across the four bands was then analyzed separately for the commensal *Enterobacteriaceae* and the non-*Enterobacteriaceae*.

RESULTS

Characteristics of the studies. Forty-six studies were identified, reporting the results for a total of 580 patients with GN bacteremia published since 1970 (Tables 1 and 2). The listed sensitivity to the internal endotoxin standard used in the assays was in the range of 0.001 to 5 ng/ml. There were 18 studies that had fewer than six patients with GN bacteremia. There was evidence suggestive of publication bias in that 10 of the 18 of studies with fewer than six patients with GN bacteremia reported that the proportion of cases of GN bacteremia that have endotoxemia was 100% (Tables 1 and 2).

Unrestricted studies. Of the 34 unrestricted studies, there were 23 with data for both commensal *Enterobacteriaceae* and non-*Enterobacteriaceae*. Among those 23 studies, the proportion of cases of GN bacteremia in each study that were commensal *Enterobacteriaceae* varied from 33% to 100% (median, 60%). From these 23 studies, an OR could be calculated for the proportion with detectable endotoxemia among those with GN bacteremia caused by commensal *Enterobacteriaceae* versus non-*Enterobacteriaceae* (Fig. 1). The summary OR was 0.53 (90% CI, 0.33 to 0.85), and there was no significant heterogeneity in the study-specific OR (chi-squared value, 8.8; $P = 0.98$; 22 df). As a sensitivity analysis, the analysis was repeated after excluding studies with fewer than six bacteremic patients, and the summary OR was 0.55 (90% CI, 0.34 to 0.9), also with no significant heterogeneity (chi-squared value, 7.8; $P = 0.96$; 16 df). Overall, of the 11 studies with data missing for either commensal *Enterobacteriaceae* or non-*Enterobacteriaceae* bac-

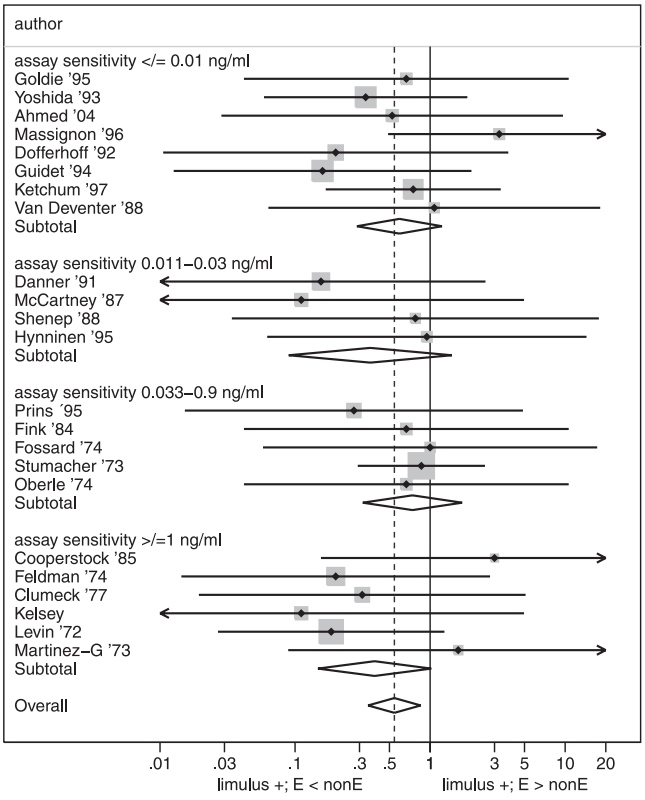

FIG. 1. Forest plot of ORs for endotoxemia with different types of GN bacteremia. The proportions of positive *Limulus* assay tests (limulus +) for patients with commensal *Enterobacteriaceae* versus non-*Enterobacteriaceae* GN bacteremia are presented as study-specific ORs and summary ORs (and 90% CIs) derived from all 23 unrestricted studies without missing data from Table 1, with studies ranked by order of *Limulus* assay sensitivity. Summary ORs for each subcategory of assay band are also displayed. Arrowheads indicate 90% CIs that extend out of range; limulus + E < nonE indicates the range in ORs for which positive *Limulus* tests are less common for bacteremia caused by *Enterobacteriaceae* than non-*Enterobacteriaceae*; limulus + E > nonE indicates the range in ORs for which positive *Limulus* tests are more common for bacteremia caused by *Enterobacteriaceae* than non-*Enterobacteriaceae*.

teremia, endotoxemia was detected in 41 of 49 (84%) cases of bacteremia (Table 1).

Restricted studies. Among 12 studies that were restricted to one of the four specified types of GN bacteremia (Table 2), the proportion with detectable endotoxemia was lowest for studies that examined *Salmonella* serovar Typhi bacteremia (four studies; 15 of 55 cases of bacteremia [27%]) compared to studies of *Neisseria meningitidis* (five studies; 69 of 84 cases of bacteremia [82%]) and *Pseudomonas pseudomallei* (one study; 38 of 41 cases of bacteremia [93%]).

Assay sensitivity. Among all 34 of the unrestricted studies, there was no significant difference in the proportion of cases with detectable endotoxemia over the bands of listed assay sensitivity for the commensal *Enterobacteriaceae* (chi-squared value, 2.27; $P = 0.52$; 3 df), although among the non-*Enterobacteriaceae*, there was a significant trend toward a higher proportion with detectable endotoxemia in the bands with the more sensitive assays (chi-squared value, 4.9; $P = 0.03$; 1 df). Repetition of the analysis after excluding studies with fewer than six cases of bacteremia reached the same conclusion (data not shown).

DISCUSSION

In this analysis of 46 clinical studies of endotoxemia diagnosis using the *Limulus* assay for cases of GN bacteremia with different types of lipid A structures, three surprising observations are noted. Among the unrestricted studies, the proportion of cases with detectable endotoxemia is lower when the GN bacterium responsible for bacteremia is a member of the commensal *Enterobacteriaceae* than when it its not a member of the *Enterobacteriaceae*. Moreover, this finding is consistent across a broad range of studies, including studies (39, 54) that reached seemingly contrary findings regarding the diagnostic utility of the *Limulus* assay with regard to endotoxemia diagnosis among cases of bacteremia caused by GN bacteria overall (Fig. 1). A second finding is that the proportion with detectable endotoxemia for cases of bacteremia caused by commensal *Enterobacteriaceae* appears to be unrelated to the sensitivity limit of the *Limulus* assay, as used in each study, over a 1,000-fold range in sensitivity to an internal endotoxin standard. Third, the proportion of cases of bacteremia with associated endotoxemia caused by GN species is highest among studies that were restricted to cases of bacteremia with *N. meningitidis* or *B. pseudomallei*, and these findings are not representative of the general diagnostic experience among unrestricted studies.

There is possible evidence of publication bias with studies that were smaller or had missing data showing atypical results. However, the conclusions of this analysis are robust regarding the inclusion or exclusion of these studies.

There are a number of limitations of this analysis. The 34 unrestricted studies were heterogeneous with respect to year of publication, patient numbers and demographics, bacteremia diagnosis and isolate identification methods, plasma extraction methods, and types of GN bacteremia. A further limitation is that the number of samples evaluated per patient in each study is not known and cannot be evaluated. Despite this heterogeneity among the studies, there is no statistical heterogeneity in the study-specific ORs among the unrestricted studies. This would not have been so readily appreciated from a either a single study in isolation or a review of the studies in the traditional narrative style without the statistical techniques of meta-analysis. Moreover, this analysis reveals that the range in the proportion of cases of bacteremia caused by commensal *Enterobacteriaceae* versus non-*Enterobacteriaceae* among the total number of cases of GN bacteremia varies widely, from 33% to 100%. This could account for the contrary conclusions regarding the diagnostic utility of the *Limulus* assay made in studies with a low (54) or high (39) proportion of commensal *Enterobacteriaceae* among the cases of GN bacteremia.

The presumption that in patients with GN sepsis, GN bacteremia and endotoxemia must always be associated with each other, even at low levels, in part contributed to earlier criticisms of the *Limulus* assay (15, 54) and stimulated the development of more sensitive versions. The findings that in other clinical settings, the concentration of endotoxin detected by the *Limulus* assay is proportional to the GN bacterial CFU count, such as in bronchoalveolar lavage samples from patients with pneumonia (50) and in urine samples of bacteruric patients (32), reinforced this presumption. However, this analysis of GN bacteremia indicates that the type of bacteremia isolate is critical to the application of the *Limulus* assay, which helps to explain why the mix of GN bacteremia isolates is influential in the concordance of GN bacteremia with the *Limulus* test of plasma (29). This is because endotoxin is undetectable for >50% of cases of bacteremia caused by the *Enterobacteriaceae*, regardless of the level of assay sensitivity.

The *Limulus* assay, a biological assay, is the most sensitive test available for the diagnosis of endotoxin (13). However, in comparisons using either the *Limulus* assay or other biological assays, the activity of endotoxin is not a uniform gravimetric property for endotoxins of different bacterial origins (12). Additionally, both the reaction kinetics (33) and the sensing of endotoxin by the *Limulus* assay (60) are affected by human plasma. It remains to be determined whether these effects of plasma differ for endotoxins from different types of GN bacteria. Moreover, the relationship between the diagnosis of endotoxemia and the clinical manifestations of sepsis has not been examined here (28, 31).

This analysis of 46 previously published clinical studies does not support the presumption that for patients with GN sepsis, GN bacteremia must always be associated with endotoxemia, but it suggests that there is a variable association of endotoxemia with GN bacteremia, which is consistent for different types of GN bacteremia.

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