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Bacterial Growth and Endotoxin Production in Lipid Emulsion

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Klebsiella pneumoniae serotypes 21 and 24 and *Enterobacter cloacae* were responsible for an outbreak of polymicrobial bacteremia associated with the receipt of lipid emulsion. Since it is recommenced that lipid emulsion be kept refrigerated between uses, we undertook a study to determine the growth characteristics of these organisms in lipid emulsion at 5 and 25°C and to examine the use of alternative measurements (pH and endotoxin) to determine contamination by viable and nonviable microorganisms. The bacteria survived but did not proliferate at 5°C; no endotoxin was detected, and the pH remained unchanged. In contrast, after a 2-h lag phase, all three organisms proliferated rapidly when incubated at 25°C and reached concentrations of $\geq 10^7$ CFU/ml at 24 h. A decrease in pH was detected after proliferation to 10^7 CFU/ml. Endotoxin was detected after proliferation reached 10^2 CFU/ml. The amount of endotoxin elaborated by the three organisms differed and ranged from 0.013 ng per 8×10^2 CFU/ml to 1.3 ng per 2×10^3 CFU/ml at 8 h. Our findings show that these microorganisms do not proliferate at refrigerator temperature in lipid emulsion, but can reach significant levels at room temperature. It is, therefore, important to keep lipid emulsion refrigerated between uses. Furthermore, when lipid emulsion contamination is suspected, endotoxin and pH determinations should be considered as possible adjunctive tests while results of bacterial cultures are pending. The results of the present study are applicable to only selected gram-negative bacteria and may not apply to gram-positive bacteria and fungi. However, these data demonstrate that measurement of pH and detection of endotoxin is quite useful when lipid emulsion contamination occurs with selected gram-negative bacteria.

The ability of intravenous solutions to support bacterial growth has been shown to depend on the nutritional components present (4). Lipid emulsion (LE) has been shown to support the growth of a number of nosocomial pathogens at 25°C (8). LE is an excellent concentrated source of energy and essential fatty acids and thus has assumed a prominent role in the supportive management of seriously ill patients. During a recent investigation, LE was epidemiologically linked to the development of polymicrobial bacteremia in a neonatal intensive care unit (6). The rapid onset of bacteremia in these infants suggested that high-level contamination of the LE had occurred. Since the manufacturers recommend that LE be kept refrigerated between uses, we sought to determine whether high-level contamination would result if artificially contaminated LE was incubated at 5 and 25°C. Furthermore, since high-level bacterial contamination of LE cannot be detected visually and the possibility exists that culturing of suspected contaminated solutions would not be productive if only nonviable microorganisms are present, we sought to evaluate whether measurement of endotoxin or pH might serve as adjunctive tests to culturing techniques when contamination of LE is suspected.

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MATERIALS AND METHODS

Bacterial strains. The microorganisms examined in this study were *Klebsiella pneumoniae* serotypes 21 and 24 and *Enterobacter cloacae*. These organisms were recovered from the blood of patients in an outbreak of polymicrobial bacteremia associated with the receipt of contaminated LE (6). These strains were chosen because they were isolated from blood cultures of infants during an outbreak, and these species have been examined in previous studies of the growth of microorganisms in intravenous solutions. A single colony of each of the three microorganisms was inoculated, individually and combined, into 5 ml of brain heart infusion broth containing 0.5% of beef extract and incubated at 35°C for 18 h. The broth-cell suspensions were concentrated and then washed by centrifugation (1,000 × *g* for 20 min) three times with 0.9% sterile NaCl. The resulting pellet of cells was then suspended in sterile saline to give a final concentration of 5 to 10 CFU/ml.

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Lipid emulsion. A 10% solution of the lipid emulsion (Liposyn; Abbott Laboratories, North Chicago, Ill.) contains the following formulation per 100 ml: 10 g of safflower oil, 1.2 g of egg phosphatides, and 2.5 g of glycerol in water for injection. The safflower oil is a mixture of neutral triglycerides of predominantly unsaturated fatty acids. The fatty acids forming the major component of the emulsion are linoleic (77%), oleic (13%), palmitic (7%), and stearic acids (2.5%).

Glass bottles containing 50 ml of LE were inoculated with 1 ml of a suspension containing 5 to 10 CFU/ml. Duplicate bottles were inoculated and incubated at 5 and 25°C. Samples were obtained at 0, 1, 2, 4, 8, 12, and 24 h for bacterial enumeration, endotoxin measurement, and pH determination. Serial 10-fold dilutions with 0.9% sterile saline were prepared for quantitative plate counts. Samples (0.1 ml) were inoculated in duplicate onto MacConkey agar plates and incubated at 36 ± 1°C for 48 h before enumeration.

pH determinations. pHs were measured with a pH meter (Corning Science Products, Medfield, Mass.). Samples (3 ml) were placed in glass tubes and frozen at 20°C until tested. At that time the samples were thawed at 25°C, and the pH was measured.

Endotoxin. Endotoxin was measured at 0, 1, 2, 4, 6, 12, and 24 h with the *Limulus* amoebocyte lysate (LAL) assay (Pyrotell; Associates of Cape Cod, Inc., Woods Hole, Mass.) (3, 5, 9). Samples (2 ml) were aseptically removed from each artificially contaminated and control bottle of LE and placed in pyrogen-free tubes for direct testing. *Esche-*

richia coli 0111:B4 lipopolysaccharide was used as an endotoxin standard control. Test samples were incubated for 1 h in a water bath at 37°C; a test result was considered positive only if the resulting clot remained firm after 180° inversion. Sterile bottles of lipid emulsion were run as negative controls.

RESULTS

All of the test strains survived, but they did not proliferate in LE at refrigerator temperature (5°C) (Fig. 1). Similarly, no significant changes in pH were detected, and the LAL tests were negative.

In the negative controls, no bacterial growth was detected, pH remained unchanged, and all LAL measurements were negative.

In contrast, all the strains tested proliferated in LE at room temperature (25°C). After a 2-h lag phase, all three organisms exhibited exponential growth to approximately 10^5 CFU/ml at 12 h. Thereafter, growth continued reaching a maximum of $\geq 10^7$ CFU/ml at 24 h.

The pH of the LE remained unchanged during the growth of all three organisms until the viable cell count reached approximately 10^7 CFU/ml. At a cell count of 10^7 CFU/ml, a decrease in pH of 0.6 units or greater was detected; decreases in pH ranged from 0.6 for *E. cloacae* to 1.5 for *K. pneumoniae* serotype 24.

Endotoxin was detectable within 4 h of incubation for *K. pneumoniae* serotype 24 and for the combined bacterial growth and after 8 h of incubation for *E. cloacae* and *K. pneumoniae* serotype 21. After 8 h of incubation, the level of endotoxin varied with the inoculated organism; endotoxin levels ranged from 0.013 ng per 8×10^2 CFU/ml for *E. cloacae* to 0.26 ng per 2×10^3 CFU/ml for *K. pneumoniae* serotype 21 to 1.3 ng per 2×10^3 CFU/ml for *K. pneumoniae* serotype 24.

DISCUSSION

The present study was limited to two gram-negative bacilli, *K. pneumoniae* and *E. cloacae*, which were recovered during an outbreak of polymicrobial bacteremia associated with the receipt of contaminated lipid emulsion (6). These organisms were selected because they have been shown to be nosocomial pathogens, and previous studies have demonstrated that these species proliferate in parenteral fluids (8). Gram-positive bacteria and fungi can also proliferate in lipid emulsion; however, only gram-negative bacteria have been implicated in previous outbreaks associated with contaminated LE (7).

Temperature is an important determinant of bacterial growth in LE. Our findings support the recommendation of the manufacturer that LE be kept refrigerated between uses. In all tests performed at 5°C, the inoculated organisms persisted but did not proliferate. In contrast, all the organisms tested at 25°C proliferated to high concentrations. These findings are consistent with those of Melly et al. (8) and Maki (D. G. Maki, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 20th, New Orleans, La., abstr. no. 533, 1980), who found that gram-negative bacilli proliferate in LE at room temperature to levels of 10^7 to 10^8 CFU/ml within 24 h.

We measured pH to see whether it might be used as a rapid indicator of LE contamination. Changes in pH were detected during bacterial growth. Although no changes in pH occurred during incubation at 5°C, changes in pH did occur when the contaminated LE was incubated at 25°C. However, it was not until bacterial growth reached approximately $\leq 10^7$ CFU/ml that significant changes in pH were detected. Thus, although pH determinations could be easily performed in hospital laboratories, such changes are not a sensitive indicator of contamination since a decrease in pH does not occur until the organisms reach high concentrations. In situations where high-level contamination is suspected, determination of pH may provide strong suggestive evidence that contamination has occurred while awaiting the results of bacterial cultures.

Since most gram-negative bacilli contain lipopolysaccharides as part of the cell wall and elaborate endotoxin during growth, measurement of endotoxin might be a rapid method to detect contamination of LE. This would provide a rapid adjunctive test while awaiting the results of bacterial cultures. Such a test would also be particularly valuable when contamination is suspected but bacteriological methods fail to recover the organism.

Endotoxin was not detected in any of the LE that was inoculated with bacteria and incubated at 5°C. In addition, all LAL tests of the uninoculated negative-control LE fluids were negative, and no false-positive reactions were detected. In contrast, endotoxin was detected in all LE contaminated and incubated at room temperature when the colony count reached 10^2 to 10^3 CFU/ml. Thus, the testing of LE fluids for endotoxin by the LAL test is sensitive and specific. Our results show that as little as 0.01 ng of endotoxin per ml can be detected and that no false-positive reactions occur. The sensitivity of the LAL test is, however, limited, and low-level contamination of LE ($<10^2$ CFU/ml or <0.01 ng of endotoxin per ml) may not be detected; this may merely reflect the fact that such small numbers of microorganisms do not elaborate endotoxin or that only minute amounts are present. Our study, therefore, demonstrates that the LAL test can be useful as an epidemiological tool in detecting contamination of LE fluids.

The present study also demonstrates that intra- and interspecies variation in endotoxin production exists within members of the family *Enterobacteriaceae*. The *Klebsiella* strains produced more endotoxin than did the *E. cloacae* strains, and *K. pneumoniae* serotype 24 produced more endotoxin than did *K. pneumoniae* serotype 21. Interspecies variation in endotoxin production has also been found with strains of *Neisseria meningitidis* (2) and *Pseudomonas aeruginosa* (1).

In conclusion, our results demonstrate that pH determination or endotoxin measurement or both can detect gram-negative bacterial contamination of LE. We did not evaluate the ability of the LAL test or pH determination to detect contamination of LE with gram-positive bacteria or fungi. Nevertheless, since only gram-negative bacterial contamination of LE has been reported, our results should be helpful to those evaluating potentially contaminated LE. Both pH determination and LAL testing are easily performed in hospital laboratories. Determination of pH is most useful in detecting high-level contamination of LE fluid. The LAL test is a very sensitive and specific test that can detect low-level endotoxin contamination of LE fluids. The present LAL test allows for direct testing of the LE without concentration or extraction. Determination of pH and use of the LAL test for the detection of endotoxin should be considered as adjunctive tests when LE contamination is suspected. With the use of these tests, rapid identification of possible LE contamination can be made and corrective action can be taken while awaiting the results of bacterial cultures.

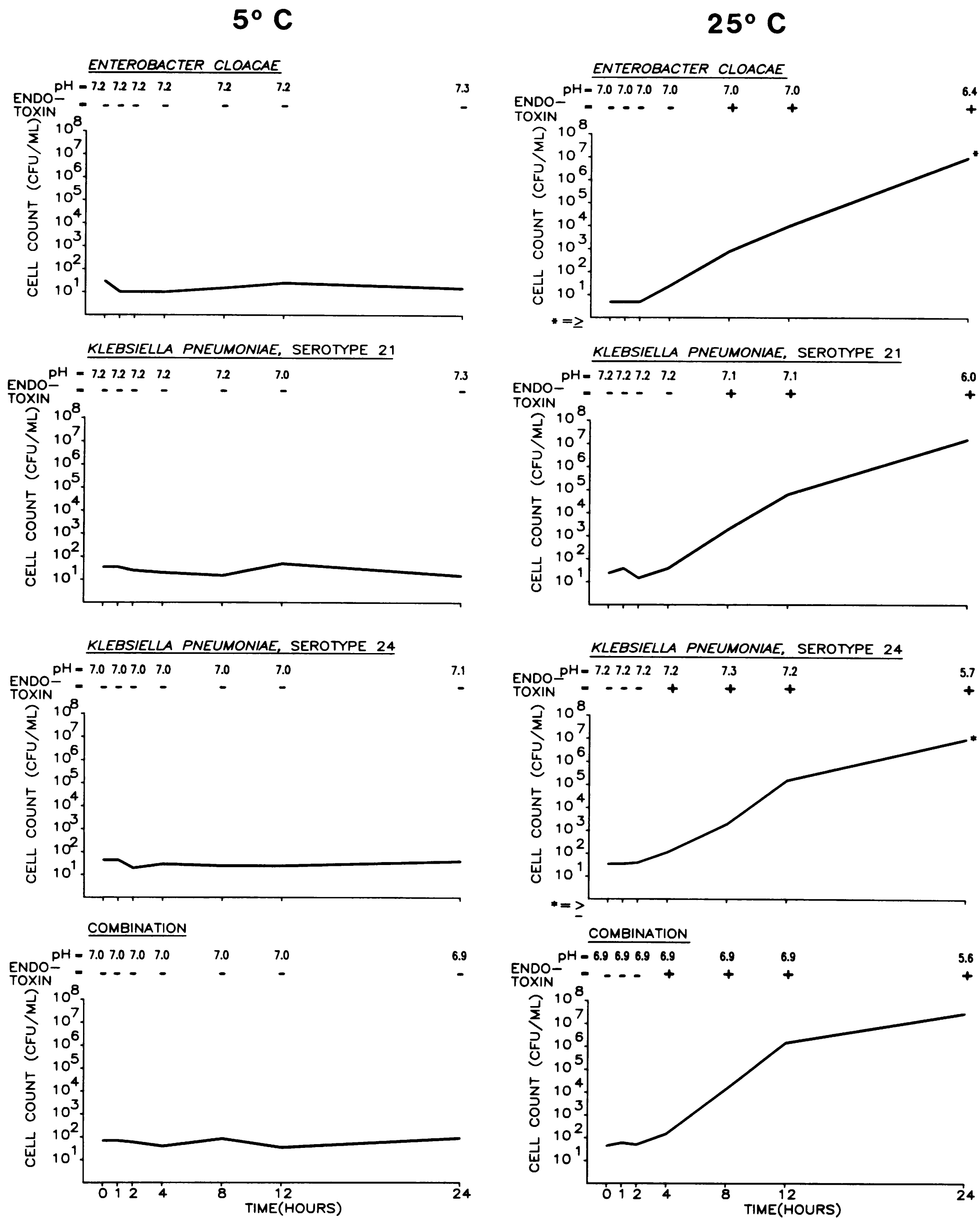

FIG. 1. Bacterial growth, endotoxin production, and pH change in lipid emulsion at 5 and 25°C.

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