

Original Article

Detection of Toxigenic Bacteria in Polyurethane Foam from Cot Mattresses by Polymerase Chain Reaction

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SUMMARY: A sensitive methodology for PCR detection of *Staphylococcus aureus* or *Bordetella pertussis* DNA within cot mattress polyurethane foam was developed. The assay's applicability was evaluated on polyurethane foam from used cot mattresses. *S. aureus* DNA was detected in 42% of mattresses of sudden infant death syndrome (SIDS) victims and 29% of comparison group (no death) mattresses tested. *B. pertussis* DNA was detected in 50% of SIDS mattresses and 27% of comparison group mattresses. There was no significant statistical association between SIDS cases and the presence of *S. aureus* or *B. pertussis* DNA in cot mattress polyurethane.

INTRODUCTION

Bacterial infections and the presence of bacterial toxins have been reported in many cases of sudden infant death syndrome (SIDS), and a common bacterial toxins hypothesis for SIDS has been proposed (1). Mattresses that have been used consecutively by more than one infant (2) and mattresses not completely covered with polyvinyl chloride (3,4) have been associated with an increased risk of SIDS. Under the common bacterial toxins hypothesis, this increase in risk could be explained by infants' exposure to toxigenic bacteria harboured within cot mattress materials. *Staphylococcus aureus* has been implicated in SIDS in a number of studies (5,6), as has *Bordetella pertussis* (7-9).

Previous studies on cot mattress microbial populations have used direct sampling for viable and culturable cells (10-14). An alternative and faster method for evaluating an infant's past exposure to toxigenic bacteria from a cot mattresses would be a PCR-based approach to detect species-specific DNA. This would have the advantage of detecting past bacterial contamination of materials, regardless of present cell viability or cell integrity status. However, no method has been described yet for the extraction and detection of DNA from polyurethane foam. The objective of this study was to establish such a methodology. We report the application of a method for assessing colonization of polyurethane foam by *S. aureus* and *B. pertussis* from SIDS and comparison (no-death) cot mattresses.

MATERIALS AND METHODS

Bacterial strains and culture conditions: Bacterial strains were obtained from the National Collection of Type Cultures. *B. pertussis* NCTC 11089 was grown on charcoal agar (Oxoid, Basingstoke, UK) containing 10% (v/v) defibrinated horse blood and 40 mg l⁻¹ cephalixin at 35°C. *S. aureus* NCTC 7447 was grown on nutrient agar (Oxoid) at 30°C.

Preparation of bacterial genomic DNA: Colonies of overnight cultures of *S. aureus*, or of 5-day cultures of *B. pertussis*, were washed in 0.1 mol l⁻¹ phosphate buffer and pelleted by centrifugation at 5,000 g for 10 min at 4°C. Genomic DNA was prepared from pelleted cells using the Genomic tip-100 kit (Qiagen, Crawley, UK) following the manufacturer's instructions.

Mattress material: Samples of new, unused cot mattress polyurethane foam (1 cm³) were excised from a square-end crib mattress (Mothercare, Leicester, UK). Each sample was seeded with 1 - 100,000 copies of target bacterial genomic DNA by inoculation with a total volume of 10 µl of diluted genomic DNA into 10 points. The number of genome copies added was calculated from each organism's genome size (data obtained from the Sanger Institute, Cambridge, UK). After inoculation, all mattress samples were incubated at room temperature for a minimum of 48 h.

Polyurethane foam mattresses (*n* = 45), collected as part of the CESDI-SUDI study during the early 1990s (15), were sampled: 24 were from SIDS cases and 21 were comparison (no-death) mattresses collected over the same period. Polyurethane foam samples (1 cm³) were taken from the top side of each mattress along its central line in the area where the infant's head would normally lie (the head-central surface).

DNA or bacterial cell extraction from cot mattress polyurethane foam: Extraction method A: Mattress material (1 cm³) was finely macerated, placed in a sterile polypropylene tube, and covered with Milli-Q Water (0.2 ml) (Millipore, Bedford, Mass., USA). The sample was then boiled for 10 min and centrifuged at 7,000 g for 30 s to pellet the mattress debris. The supernatant was decanted and stored at -20°C prior to use.

Extraction method B: Mattress material (1 cm³) was ground into a fine powder under liquid nitrogen, transferred to a sterile polypropylene tube, and covered with Milli-Q Water (0.2 ml), and thereafter treated as in extraction method A.

Extraction method C: Mattress material (1 cm³) was finely macerated, placed in a sterile polypropylene tube, and covered with 0.2 ml buffer A (50 mmol l⁻¹ Tris HCl, 20 mmol l⁻¹ EDTA, 100 mmol l⁻¹ NaCl, 1% [w/v] sodium dodecyl sulphate, pH 8.0). Samples were incubated at 70°C for 20 min with periodic mixing, after which they were centrifuged at 7,000 g for 30 s to pellet the mattress debris. The supernatant was decanted and the extracted DNA ethanol precipitated in

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the presence of 20 μg molecular biology-grade glycogen (Invitrogen, Glasgow, UK). The resulting pellet was re-suspended in 25 μl Milli-Q Water and stored at -20°C prior to use.

Extraction method D: Mattress material (1 cm^3) was finely macerated, placed in a sterile polypropylene tube, and covered with 0.2 ml Milli-Q Water. The samples were subjected to five freeze-thaw cycles in which they were snap-frozen in liquid nitrogen and then thawed in a boiling waterbath. After centrifugation at 7,000 g for 30 s, the supernatant was decanted and stored at -20°C prior to use.

Extraction method E: Mattress material (1 cm^3) was finely macerated, placed in a sterile polypropylene tube, and covered with 0.2 ml Milli-Q Water. Samples were placed on ice and subjected to sonication using an MSE Soniprep 150 ultrasonic disintegrator (MSE Scientific Instruments, Crawley, UK) at an amplitude of 12 μm for 3 min. After centrifugation at 7,000 g for 30 s, the supernatant was decanted and stored at -20°C prior to use.

PCR: PCR primers (Invitrogen) targeted either a 924-bp region of the *S. aureus* *SigB* gene (SAF, 5'-ATGGCGAAAGA GTCGAAATC -3'; SAR, 5'-CGCGACATTTATGTGGATACAC -3'; GenBank gene identifier, 1729791; [16]) or a 406-bp region of the promoter sequence of the *B. pertussis* S1 subunit of the pertussis toxin gene (BPF, 5'-TGCAGATTCGT CGTACAAAACCCT-3'; BPR, 5'-AACGTCCGGTCAGAT GGTCGAGCA-3'; GenBank gene identifier, 3152312; [17]). The primer pair employed for the detection of *Bordetella* in this study allowed the specific detection of *B. pertussis*, as the *B. parapertussis* genome differs in sequence in the region used to design the primers (17). PCR reactions were assembled as a mastermix of common reagents and included 0.3 $\mu\text{mol l}^{-1}$ of each gene-specific forward and reverse primer, 200 $\mu\text{mol l}^{-1}$ deoxyribonucleoside triphosphates (Invitrogen), and BIOTAQ reaction buffer (Bioline, London, UK; 16 mmol l^{-1} $(\text{NH}_4)_2\text{SO}_4$, 67 mmol l^{-1} Tris-HCl [pH 8.8 at 25°C], 0.01 % [v/v] Tween-20, 1.5 mmol l^{-1} MgCl_2). After the volume was adjusted to 30 μl with Milli-Q Water, 20 μl of the template, either mattress-extracted material or an appropriate control sample, was added to each reaction mixture. PCR amplification was performed in an MJ Research thermal cycler (Genetic Research Instrumentation, Braintree, UK) for a total of 40 cycles. The first cycle was preceded by a 5 min denaturation at 94°C during which 2.5 units of BIOTAQ was added to each tube. Each cycle consisted of denaturation at 94°C for 45 s, 30 s at an annealing temperature of 55°C , and 45 s of extension at 72°C . The final cycle was followed by an extension at 72°C for 5 min. Post-amplification, 10 μl of each reaction product was analysed by electrophoresis on a 1% (w/v) agarose gel in Tris-Borate-EDTA buffer (pH 8). The gel was stained with 0.5 $\mu\text{g ml}^{-1}$ ethidium bromide (Sigma, Poole, UK) and images were obtained after illumination with short UV light using a gel documentation system (Syngene, Cambridge, UK). Amplicon sizes were estimated by comparison with the molecular weight marker 1 Kb ladder (Invitrogen).

Assessment of sensitivity and quality control: To determine the analytical sensitivity of the extraction procedures coupled with the PCR detection assay, foam samples were seeded with 1, 5, 10, 50, 100, 500, 1000, 10,000, and 100,000 genome copies or viable cells per 1 cm^3 , extracted, and subjected to PCR. All assays were performed according to standard quality assurance guidelines for molecular diagnostics (18). For example, to assess for PCR inhibitors in the foam-

extracted material, bacterial genomic DNA was amplified in both the presence and absence of mock extracted samples (i.e., extracted in the absence of added DNA). Furthermore, for the study involving SIDS and the comparison group (no-death) mattresses, each PCR experiment ($n > 3$) contained: (i) a positive control amplification of genomic DNA; (ii) a reagent contamination control (no added template) amplification; (iii) a reaction using material extracted from an unused mattress seeded with the DNA of the bacteria under investigation; and (iv) a reaction using material extracted from an unseeded, unused mattress. The last two controls were extracted alongside the sample mattresses.

Statistical analysis of data: Samples were grouped according to frequency of detection for individual bacterial species. Estimates and 95% confidence intervals for the odds ratios were calculated, and the frequency distribution (P -value) for data sets was calculated using Fisher's Exact Test (expected frequency < 5).

RESULTS

The amplification primers for the detection of *S. aureus* and *B. pertussis* had no significant homology with sequences other than the target sequences deposited at GenBank, as ascertained by BLAST homology searches, and they did not produce an amplification product when human genomic DNA was used as a template for PCR. When tested on genomic DNA of the bacteria from which they were derived, each primer set amplified a DNA species of the predicted size, 924 bp (Fig. 1A lane ii) and 406 bp (Fig. 1B lane ii) for *S.*

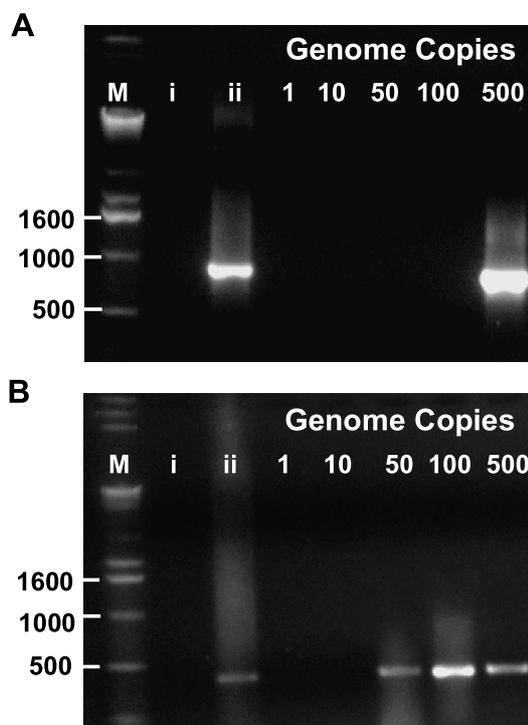


Fig. 1. Evaluation of the limit of detection of bacterial DNA presence in cot mattress polyurethane foam. Photograph of an agarose gel electrophoresis of the amplicons resulting from PCR (40 cycles) with the relevant species-specific primers of extracted specimens from cot mattress polyurethane foam by method A after inoculation with the indicated genome copies of genomic DNA (A) *S. aureus* or (B) *B. pertussis*. In both (A) and (B), lane i contains an amplification from an extraction of an uninoculated mattress sample and lane ii an amplification from the respective genomic DNA. Marker (M), 1 Kb ladder.

aureus and *B. pertussis*, respectively. The identity of each amplification product was confirmed by sequence analysis of the purified PCR products; both were identical to the previously reported sequences (16,17).

The extraction methods were tested using *S. aureus* as a model system. Foam samples, previously seeded with either 1×10^4 or 1×10^5 genome copies of *S. aureus* genomic DNA, were subjected to extraction using the five procedures described in Methods. Of the five methods tested, material obtained from methods A and B gave positive results when used as PCR templates, whereas methods C, D, and E did not produce amplifiable material.

The sensitivity of extraction methods A and B was assessed for *S. aureus* by seeding foam samples with serially diluted genomic DNA and subjecting 20 μ l of the eluate to PCR. The detection limit was lower for method A at 500 genome equivalents per cm^3 (Fig. 1A) than for method B at 1,000 genome equivalents per cm^3 (data not shown). This corresponds to approximately 50 genome equivalents per reaction if 100% recovery during DNA extraction is assumed. The sensitivity of method A was also assessed for the *B. pertussis* assay. In this case the limit of sensitivity was 10 genome equivalents per cm^3 (Fig. 1B). This equates to approximately 1 genome equivalent per reaction, again assuming 100%

recovery of DNA during the extraction procedure. In each case, there was no detectable amplification from uninoculated mattress samples. The sensitivity of extraction method A was also assessed by seeding foam samples with serially diluted cell suspensions of *S. aureus* or *B. pertussis*. In comparison with corresponding sensitivities for purified DNA, that for *S. aureus* cells was approximately threefold lower (1,500 cells cm^{-3}), while that for *B. pertussis* cells was approximately 10-fold lower (100 cells cm^{-3}).

The simplicity of method A, coupled with its greater sensitivity, made it the method of choice for the extraction of DNA from polyurethane foams. Qualitative analysis of the colonization of 45 previously used mattresses by *S. aureus* and *B. pertussis* was then performed. Material was extracted from the CESDI-SUDI mattresses at the head-center surface region alongside assay control samples and was subjected to PCR employing the *S. aureus* and *B. pertussis* primer sets ($n > 3$). A representative experiment for *S. aureus* is presented in Figure 2, and qualitative data for the PCR detection of both species in these mattresses is summarized in Table 1. No amplification of either species was apparent from unused mattresses assayed in parallel. Twofold dilution of the extracted material prior to PCR also failed to generate amplification products, suggesting that the level of bacterial contamination approached the assay's sensitivity limit. There was no significant difference in the frequency of detection of *S. aureus* or *B. pertussis* in polyurethane foam between SIDS and comparison (no-death) mattresses.

DISCUSSION

The detection limits for PCR-based bacterial screening methods dependent usually on the efficiency of the DNA extraction method used. This has previously been reported for the detection of pathogens in food (19) but has not been investigated with regard to polyurethane foam. The most sensitive of the five methods used to extract DNA from cot mattress polyurethane foam was method A, in which the sample was macerated prior to boiling. The only other protocol that produced amplifiable DNA was method B, in which the sample was ground to a fine powder under liquid nitrogen prior to boiling. Failure to generate amplicons from the other extraction methods tested could be a consequence of their potentially lower sensitivity or could be due to parallel extraction of an inhibitor of the PCR. Although hot detergent washing, freeze-thawing, and sonication methods have been applied to soil (20), sediments (21), and caecal lumen (22) with varying degrees of success, we found that these methods were not suitable for extracting DNA from polyurethane foam. This may be due to the physical properties of the polyurethane foam or to limitations inherent to the method. For example, the failure of repeated freeze-thawing to have a significant effect on polyurethane foam may be due to its homogeneity and elasticity. Similarly, the porous high surface area of the foam could reduce the efficiency of washing methods. Conversely, sonication is reported to shear DNA, yielding fragments of 100-500 bp (23), so the lack of detection could be due to degradation of DNA by this extraction method.

We detected *S. aureus* and *B. pertussis* DNA in polyurethane foam from cot mattresses used in the CESDI-SUDI study. The overall detection frequency (36%) of *S. aureus* in polyurethane foam from these used mattresses was lower than that (60%) in foam from mattresses in more recent regular use and as assessed by the viable counting method (13).

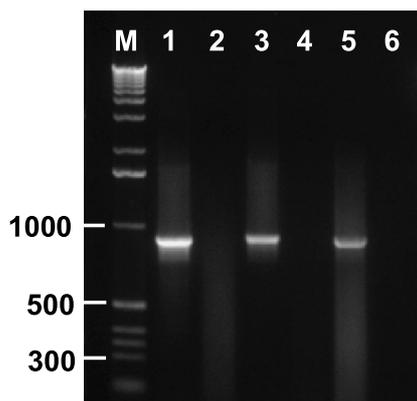


Fig. 2. Qualitative analysis of *S. aureus* colonization of polyurethane foam from cot mattresses by PCR. Photograph of an agarose gel electrophoresis of the amplicons resulting from PCR (40 cycles) employing the *S. aureus* primer set using the following template sources: lane 1, *S. aureus* genomic DNA; lane 2, no added template control; lane 3, extraction from an inoculated mattress sample, seeded with 1,000 genome copies of *S. aureus* DNA; lane 4, extraction from an uninoculated mattress sample; lane 5, extraction from a *S. aureus* positive CESDI-SUDI study mattress sample; and lane 6, extraction from a *S. aureus* negative CESDI-SUDI study mattress sample. Marker (M), 1 Kb ladder.

Table 1. PCR detection of *S. aureus* and *B. pertussis* DNA isolated from polyurethane foam of used cot mattresses

Organism	Frequency of detection		<i>P</i>
	Comparison group (no-death)	SIDS	
<i>S. aureus</i>	<i>n</i> = 21	<i>n</i> = 24	0.53
	6 (29)	10 (42)	
	OR 1.0	1.8, CI 0.5 to 6.2	
<i>B. pertussis</i>	<i>n</i> = 15	<i>n</i> = 14	0.26
	4 (27)	7 (50)	
	OR 1.0	1.7, CI 0.4 to 7.1	

Values in parentheses are numbers of positive samples expressed as percentages.

OR, CI, 95% confidence intervals for odds ratio.

Degradation of bacterial DNA in CESDI-SUDI mattresses since collection (ca. 10 years earlier) by ubiquitous nucleases may account for this lower detection frequency. We have isolated colonies typical of *B. pertussis* on charcoal blood agar plates (containing cephalixin) at a frequency of 17% in polyurethane foam from used cot mattresses in regular use (13); subsequent loss of the isolate during subculture precluded presumptive identification to the species level and is consistent with the established poor environmental survival of *B. pertussis*. The far higher frequency PCR-based detection (38% overall, reported here) of *B. pertussis* in used cot mattress polyurethane foam is again consistent with its poor environmental survival and highlights the need for a noncultivation-based approach to estimate past contamination of materials with this bacterial species. We note that the whole-cell pertussis vaccine was used in the UK throughout the period of mattress collection, and we cannot exclude the possibility that this intramuscularly injected, killed bacterial suspension is a source of *B. pertussis* DNA within cot mattresses.

Statistical comparison of the frequencies of PCR-based detection of *S. aureus* or *B. pertussis* between the SIDS and comparison (no-death) group mattresses did not provide evidence that their presence in cot mattress polyurethane foam had a causal relation to SIDS. However, an unambiguous link between SIDS and bacterial contamination of cot mattresses was unlikely due to the small sample size, potential DNA degradation during the length of time between mattress collection and sampling (ca. 10 years), and the nature of the populations sampled. Current evidence indicates that not all infants would be susceptible to SIDS, possibly due to a genetic factor (24,25). In this case, contaminated mattresses would be included in the non-SIDS category. Similarly, a SIDS trigger event would lead to infant death only if it occurred within a period of vulnerability during infant development. Mattresses from a susceptible household that were contaminated outside this risk period would also be included in the non-SIDS category. Conversely, SIDS events resulting from exposure to a trigger event not associated with a cot mattress, or involving a microorganism that was not assayed for, would result in the inclusion of uncontaminated mattresses in the SIDS category. To more thoroughly assess the possible contribution of toxigenic bacteria within cot mattresses, a study incorporating a larger sample size and methods for detecting other bacteria implicated in SIDS, such as *Clostridium perfringens* and *Escherichia coli*, should be performed.

In conclusion, this study has shown that it is possible to extract bacterial DNA from polyurethane foam and that a proportion of cot mattresses become contaminated with the potentially harmful bacteria *S. aureus* and *B. pertussis* upon use.

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