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Evaluation of the growth of microorganisms on diaper absorbent materials

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SUMMARY

Methods were developed to study the effects of absorbent materials from diapers on microbial survival, growth and toxic shock syndrome toxin-1 (TSST-1) production under specified in vitro conditions. Growth of representative skin and fecal flora organisms was equivalent in cultures in which materials from cotton cloth diapers, disposable diapers or disposable diapers containing absorbent gelling material were added as the sole carbon source. In urine used as an enrichment medium, growth of the test organisms in media containing material from the three diaper types was equivalent and no contribution to growth from the diaper material was detected. TSST-1 was not produced by *Staphylococcus aureus* under conditions in which urine was added to the diaper materials. Pathogenic strains of organisms purposefully introduced onto diapers failed to survive and the few microbial cells normally found in diaper material did not multiply when stored under conditions favorable to microbial growth. The data indicate that all three diaper types tested were the same with respect to growth and survival of representative skin and fecal organisms.

INTRODUCTION

The skin microflora of young children is varied and is influenced by age, hygiene, pH, moisture, soiling and other factors [1,7,11–13]. Variations in skin moisture and the availability of nutrients are cited as the major reason for differences in skin flora. Diapers can influence the skin microflora by affecting skin moisture and by maintaining urine and feces in close proximity to the skin. For example, the flora on skin under diapers is known to be increased in number, as compared to skin not covered by a diaper [1,7,9,11–13].

Diapers are designed to hygienically catch and hold urine and feces voided by children until the diaper can be changed. All diapers utilize absorbent materials which have traditionally been made of cotton cloth (cellulose fibers) or wood pulp (cellulose). Recently, absorbent gelling materials (AGM) have been added to conventional wood pulp cellulose diapers to improve their urine-holding capability, which results in improved skin condition and diaper rash control [2–4,8,15,17].

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Although diapers may influence the microorganisms found on skin [7], the interaction of the materials used to make diapers with skin and fecal flora has not been thoroughly studied. This paper reports on studies which investigated the effects, under in vitro experimental conditions, of various absorbent materials used in diapers on microorganisms typically found on the skin and in the feces of diapered children. Methods were developed and used to evaluate the effect of various diaper absorbent materials on the in vitro survival, growth and toxic shock syndrome toxin-1 (TSST-1) production of microorganisms endogenous to the normal skin and feces.

MATERIALS AND METHODS

Diapers

Diaper component materials tested were cellulose wood pulp, cotton cloth and AGM.

Diapers studied were a disposable diaper containing standard cellulose core material (Pampers; Procter and Gamble Co., Cincinnati, OH), cotton cloth (Curity; Kendall Co., Boston, MA) and a disposable diaper containing a cellulose core with AGM (Ultra Pampers: Procter and Gamble Co.).

Microorganisms

For initial studies in chemically defined media, microorganisms were obtained from the American Type Culture Collection: *Staphylococcus aureus* ATCC E6538, *S. aureus* ATCC 6538P, *Escherichia coli* ATCC E8739, *Proteus vulgaris* ATCC E33420, *Pseudomonas aeruginosa* ATCC 9027, and *Candida albicans* ATCC 10231. Additional organisms used for the persistence study were clinical isolates of *E. coli* and *C. albicans,* and *P. aeruginosa* ATCC 27853. Clinical isolates (obtained from Cincinnati Children's Hospital) of *E. coli, S. aureus, S. epidermidis, C. albicans* and *Pr. mirabilis* were used in studies where urine was used as culture medium. *S. aureus* strain FRI1169 was obtained from Dr. J. Kirkland of The Procter and Gamble Company.

Media

A chemically defined medium without a carbon or nitrogen source [6] was used in the initial growth studies. The medium was supplemented with Basal Medium Eagle vitamins (Flow Laboratories, McLean, VA). *C. albicans* and *S. aureus* required a supplement of 1.0% and 0.005% Bacto-peptone (Difco Laboratories, Detroit, MI), respectively, to maintain viability. *C. albicans* and *Pr. vulgaris* additionally required a nitrogen source which was supplied as 0.1% (w/v) ammonium sulfate. For experiments using clinical microbial isolates, urine used as growth medium was collected from 12 children (1–4 years old), pooled, and filter-sterilized through a 0.45 μm pore size membrane filter. Urine was kept frozen until used.

Trypticase soy agar (BBL Microbiology Systems, Cockeysville, MD), tergitol 7 agar (BBL), mannitol salt agar (BBL), Todd-Hewitt broth (Difco), and Sabouraud dextrose agar (BBL) containing 0.05 g/l chloramphenicol (Sigma Chemical Co., St. Louis, MO) and 0.05 g/l gentamicin (Sigma) were used to assay specific organisms as appropriate.

Experimental design

Growth studies in chemically defined media

Cultures of each organism were prepared from overnight cultures by suspending the cells in phosphate-buffered saline (PBS; pH 7.0) [16] at approximately 10^8 cfu/ml by comparing the density visually with a 0.5 McFarland standard. The starting culture was diluted to remove overnight medium and added to the chemically defined medium without a carbon source to yield a starting inoculum of approximately 10^3 cfu/ml. Diaper core absorbent materials were added to the medium at 0.5% (w/v). All cultures were 100 ml volumes in 250 ml flasks which were incubated at 37°C on a rotary shaker at approximately 200 rpm.

The actual zero-hour cell concentration was determined by plating an aliquot from the inoculated base medium on trypticase soy agar (TSA). Growth in all cultures was quantified following 5 and 24 h incubation by plating on TSA.

Growth of clinical isolates in urine

Starting cultures of each organism were prepared from overnight cultures of the organism at approximately 10^8 cfu/ml in PBS by visual comparison with a 0.5 McFarland standard. The starting culture was diluted and added to the urine to yield a starting inoculum of approximately 10^3 cfu/ml. Two swatches (5.1 cm in diameter) of each diaper type were placed individually in sterile 250 ml flasks and inoculated with a volume of 20–26 ml, based on ten times the weight of the swatch, and incubated on a rotary shaker at 37°C. At 5 and 24 h post-inoculation, each swatch was homogenized in a blender with 150 ml of PBS, pH 7.0. The homogenate was diluted and plated by the spiral plating method (Spiral Systems, Bethesda, MD) onto appropriate media. Following incubation at 37°C for 24–48 h, the colonies were enumerated and the cfu/ml of each culture was determined. Five and 24 h represent typical daytime and maximum practiced wearing periods for diapers.

Growth of TSST-1-producing strain of S. aureus (FRI1169)

Diaper swatches were prepared aseptically by using a sterile metal die to cut 5.1-cm-diameter full-thickness cores from the diapers. Three swatches were placed individually in sterile 250 ml flasks and inoculated with 10^7 cfu/ml of *S. aureus* and 10^5 cfu/ml of *E. coli* ATCC E8739 in urine. A culture in Todd-Hewitt broth was included as a positive control. Preliminary studies showed that no toxin was produced in the control cultures under stationary conditions, therefore cultures were agitated to maximize the potential for toxin production [14].

The zero-hour concentrations of *S. aureus* and *E. coli* were determined by plating 0.1 ml of tenfold dilutions of the original cell suspension onto TSA. After incubation, two swatches were homogenized in a blender with 300 ml of PBS and the homogenate was quantified. Aliquots (0.1 ml) of appropriate dilutions in PBS pH 7.0 were spread on appropriate selective media. Each dilution was plated in duplicate and, after an incubation period of 24 h, those plates which contained 30–300 colonies were counted.

Additionally, culture fluid was expressed from a third swatch, removed from the flask and centrifuged at 6000 × *g* for 10 min to remove cell debris. TSST-1 was assayed by the microprecipitin assay described by Fung and Wagner [5]. Toxin banding was quantified by comparison with a standard curve prepared using a TSST-1 standard obtained from Toxin Technology (Madison, WI). The antiserum used was sheep anti-TSST-1 obtained from Dr. J. Kirkland.

Persistence of microorganisms in diapers

Diaper swatches (diameter 5.1 cm) were cut from each of the diaper types, sterilized by ethylene oxide gas and aerated under ambient conditions to remove residual sterilant. Starting cultures of each organism were prepared from overnight cultures of the organisms at approximately 10^9 cfu/ml in PBS by visual comparison with a 5 McFarland standard. The starting culture was diluted to remove overnight culture medium and added to the PBS to yield a starting inoculum of approximately 10^6 cfu/ml. After inoculation the test swatches were placed in labeled plastic trays. The trays were covered with a non-woven surgical drape to allow passage of air and moisture, but not air-borne contamination, and stored at 27°C and 80% relative humidity. Immediately following inoculation, and at 1, 3, 6 and 14 days following inoculation, three diaper swatches per product type and per organism were assayed. Additional platings were performed on days 35 and 69 for *S. aureus*- and *E. coli*-inoculated swatches, on days 28 and 92 for *P. aeruginosa,* and on days 28 and 55 for *C. albicans.*

To determine the numbers of surviving organisms, swatches were homogenized in a blender with 300 ml of PBS and the homogenate was plated in duplicate on the appropriate media. For samples expected to have low numbers of organisms, three swatches were overlaid with TSA. Following incubation at 37°C for 48–72 h, the colonies were enumerated.

Bioburden assay

Six diapers of each type were placed aseptically in cartons and stored in an environmentally con-

trolled room at 27°C and 80% relative humidity. At zero time and after 1, 6, 12 and 24 weeks, a carton was removed and the diapers were analyzed for bioburden. Using a sterile metal dye, a 5.1-cm-diameter swatch of the respective diaper was cut from the diaper, placed in a sterile petri dish and saturated with TSA. Following incubation for 48 h at 37°C, the number of colonies on the diaper was enumerated and the cfu/g of diaper was calculated.

RESULTS

Growth assays in defined medium

The results of growth experiments in a defined medium which included diaper material as the only potential carbon source (Table 1) demonstrate that the growth of the test organisms was equivalent in each of the diaper materials tested, as all were within 2 $\log_{10}$ cfu/ml of each other (95% confidence limit).

Table 1

Growth of microorganisms inoculated in chemically defined media without a carbon source

Diaper materials were tested at 0.5% (w/v) in a defined growth medium without a carbon or nitrogen source and incubated for either 5 or 24 h at 37°C. Values are the means of duplicate experiments.

Organism	Diaper	$\log_{10}$ cfu/ml at incubation time (h):		Log change from 0 h at incubation time (h):	
		5	24	5	24
S. aureus	none	3.1	6.2	−0.2	2.9
	disposable	3.0	4.7	−0.2	1.4
	cloth	2.3	4.5	−1.1	1.3
	AGM	3.4	4.8	−0.4	1.4
E. coli	none	3.4	4.6	0.2	1.2
	disposable	3.6	5.3	0.0	1.8
	cloth	3.1	5.1	−0.5	1.6
	AGM	3.6	5.6	−0.4	2.1
Pr. vulgaris	none	3.7	3.5	0.1	−0.1
	disposable	3.7	4.1	0.1	0.6
	cloth	3.6	5.1	0.1	1.5
	AGM	3.6	4.5	0.0	0.8
C. albicans	none	3.6	5.2	−0.2	1.4
	disposable	3.4	6.0	−0.4	2.2
	cloth	2.9	5.3	0.1	1.5
	AGM	3.7	4.5	−0.1	0.7

Growth of clinical isolates in urine

While growth assays in defined medium are a valuable screening technique, they do not duplicate the actual diaper environment, which includes nutrient-rich urine and feces. Therefore, to produce an environment that is richer in nutrients, urine was employed to study the growth of clinical isolates of *S. aureus, S. epidermidis, E. coli, C. albicans* and *Pr.*

Table 2

Growth of clinical isolates in urine

Cultures were inoculated with an initial population of approximately 10^3–10^4 organisms/ml in a volume based on ten times the weight of the swatch and incubated on a rotary shaker at 200 rpm and 37°C.

Organism	Diaper	Log change from 0 h at incubation time (h):	
		4	24
S. aureus	none	0.9	5.2
	disposable	0.3	5.4
	cloth	0.4	5.1
	AGM	0.2	5.1
S. epidermidis	none	−0.1	5.9
	disposable	−0.4	5.7
	cloth	0.3	5.8
	AGM	−0.2	4.4
E. coli	none	1.6	5.6
	disposable	0.8	5.7
	cloth	1.4	5.6
	AGM	1.6	5.8
Pr. mirabilis	none	0.6	3.9[a]
	disposable	0.5	4.1[a]
	cloth	0.8	4.2[a]
	AGM	0.8	4.6[a]
C. albicans	none	0.1	3.4
	disposable	0.6	4.0
	cloth	0.4	3.9
	AGM	0.4	4.2

[a] Log change from 0 h at 10 h incubation (see text).

mirabilis. Under these conditions, the growth of each organism was equivalent in medium containing each of the diaper types (Table 2). Although comparable maximum growth (4.1–5.0 log units) of *Pr. mirabilis* was achieved in each of the cultures, the maximum growth was reached by 10 h in cultures with cloth diapers as compared to 10–14 h for the other diaper types. Maximum growth of *Pr. mirabilis* was followed by rapid die-off as cultures became increasingly alkaline. These data indicate that, compared to control cultures, there was no detectable contribution of the diaper materials to the growth of any of the test organisms.

Growth and TSST-1 production by S. aureus strain FRI1169

Growth and production of TSST-1 by *S. aureus* strain FRI1169 was studied. Filter-sterilized urine was used as a medium and *E. coli* was included as an additional representative of the skin and fecal flora. Cultures were incubated at 37°C and 300 rpm for 24 h. Cultures of all diaper types yielded equivalent levels of growth for both organisms under the simulated diaper conditions (Table 3). Growth of *S. aureus* and *E. coli* reached 9.1–9.5 $\log_{10}$ cfu/ml and 8.8–9.1 $\log_{10}$ cfu/ml, respectively. TSST-1 was not detected in any cultures containing diaper material or in the urine control. TSST-1 production was observed in the positive control (THB), confirming the TSST-1-producing capability of the strain. Separate studies (not shown) established that the addition of urine to cultures of *S. aureus* reduced TSST-1 production. No TSST-1 was detected in cultures grown in 100% urine; 50% urine in THB reduced TSST-1 production by 47%. Additionally, TSST-1 was not detected in cultures of *S. aureus* and *E. coli* in THB or *S. aureus* and *E. coli* in a 50% mixture of THB and urine. The detection limit of the assay for TSST-1 is 3 μg/ml.

Persistence study

It is possible that diapers could be contaminated with organisms during manufacture and consumer use. To determine whether organisms accidentally introduced onto diapers are able to survive and utilize the diaper materials to grow, the survival of *E. coli, S. aureus, C. albicans* and *P. aeruginosa* was studied on diaper swatches stored under conditions favorable to survival (80% relative humidity and 27°C). None of the organisms was detected at 14 days or beyond in the AGM diapers (Table 4). Cloth diapers allowed survival of all organisms after 14 days of storage and *E. coli* was detected at 14 days in the disposable diapers.

Table 3

Growth and TSST-1 production by *S. aureus* strain FRI1169 in diaper swatches

Diaper	Medium	$\log_{10}$ cfu/ml at 24 h		Log increase in growth at 24 h		TSST-1 (μ/ml)
		S. aureus[a]	*E. coli*[b]	*S. aureus*[a]	*E. coli*[b]	
No product	THB	9.5	–[c]	2.6	–[c]	16.4
No product	urine	9.5	9.1	2.4	3.9	n.d.[d]
Cloth	urine	9.2	9.0	2.1	3.7	n.d.
Disposable	urine	9.1	8.9	2.0	3.6	n.d.
AGM	urine	9.1	8.8	2.0	3.6	n.d.

[a] Initial inoculum 10^7 cfu/ml.

[b] Initial inoculum 10^5 cfu/ml.

[c] No *E. coli* included in cultures.

[d] n.d. = none detected. The detection limit of the assay is 3 μg/ml. Values are means of duplicate cultures from each of four replicate experiments.

Table 4

Survival of microorganisms in diapers stored at 80% relative humidity and 27°C

Diaper swatches (5.1 cm diameter) were inoculated with approximately 10^6 cfu/swatch of the test organism and placed in storage at 80% relative humidity and 27°C for up to 92 days. n.d. = not detected. Values are the means of triplicate determination.

Organism	Diaper	Log_{10} cfu/swatch at day of storage:						
		0	1	3	6	14	28–35	55–92
S. aureus	disposable	6.1	5.5	4.0	<1.0	n.d.	n.d.[a]	–
	cloth	6.2	5.9	5.1	2.7	0.7	n.d.[a]	n.d.[b]
	AGM	6.2	5.8	4.4	<1.0	n.d.	n.d.[a]	–
E. coli	disposable	6.5	4.2	<2.0	2.7	1.0	n.d.[a]	n.d.[b]
	cloth	6.5	5.9	5.7	3.8	1.4	n.d.[a]	0.4[b]
	AGM	6.6	5.5	5.8	2.9	n.d.	n.d.[a]	n.d.[b]
P. aeruginosa	disposable	6.9	3.5	<2.0	<1.0	n.d.	n.d.[c]	–
	cloth	6.6	4.4	4.5	4.3	2.0	1.4[c]	n.d.[d]
	AGM	6.6	4.3	<2.0	<1.0	n.d.	n.d.[c]	–
C. albicans	disposable	5.6	5.3	4.5	3.9	n.d.	n.d.[c]	n.d.[c]
	cloth	5.8	5.8	5.9	5.6	4.7	n.d.[c]	n.d.[e]
	AGM	5.8	5.3	3.7	1.8	n.d.	n.d.[c]	–

[a] 35 days; [b] 69 days; [c] 28 days; [d] 92 days; [e] 55 days.

Bioburden study

Since microorganisms are normally present in cellulose fibers in either cotton cloth or wood pulp, the ability of the naturally occurring bioburden organisms to survive and grow was examined on diapers stored under warm, moist conditions. Bioburden determinations were made on each diaper type at intervals up to 24 weeks of storage. The bioburden of each test diaper was low and the numbers of organisms declined during storage (Table 5). Other studies (data not shown) demonstrated a consistently low bioburden for disposable diapers. Separate testing demonstrated that the AGM material itself did not contain a detectable bioburden population of organisms.

Table 5

Bioburden survival on diapers stored at 80% relative humidity and 27°C

Diapers were stored in cartons in an environmentally controlled room for up to 6 months. The bioburden was assayed by agar overlay. Values are the means of triplicate determinations.

Diaper	cfu/g at storage week:				
	0	1	6	12	24
Disposable	4.1	3.8	3.8	2.9	2.4
AGM disposable	9.1	2.9	2.0	2.7	1.7
Cloth	13.8	4.9	6.7	2.2	0.4

DISCUSSION

Results of the in vitro growth assays conducted in a chemically defined minimal medium clearly demonstrate that the limited growth of the test organisms was equivalent in each of the diaper materials tested when the diaper materials were the only substrate present.

The lack of effect of a diaper material on the growth of skin and fecal microorganisms was further confirmed under nutrient-rich conditions in

urine-containing cultures. None of the diaper materials supported detectable microbial growth in excess of that in the urine alone. These results are equivalent to those obtained from studies of mixed cultures reported previously [9]. Together, the results from the defined medium and enrichment medium (urine) studies suggest that, under in vivo conditions where nutrients are readily available, the diaper materials themselves would not contribute to the growth of skin and fecal microorganisms. This conclusion is further supported by the results of clinical studies of the skin of children wearing diapers, which clearly demonstrate that the skin flora of children wearing different diaper types is equivalent [9,15].

None of the diaper materials tested differed in their effects on the growth of *S. aureus*. The results also demonstrate that the presence of urine and feces (*E. coli*), that are often present in a diaper, inhibit the production of TSST-1 by a strain of *S. aureus* which can produce the toxin.

Since the materials used in diapers are absorbent and since moisture and nutrients control microbial growth and survival, the persistence of microorganisms in each of the diaper materials was studied. No test organisms survived on AGM diapers at 14 days and only *E. coli* was detected at 14 days on disposable diapers. In contrast, all four test organisms survived in cloth diapers after 2 weeks of storage. Only *P. aeruginosa* remained viable after 4 weeks of storage on cloth diapers. This suggests that even if organisms were accidentally introduced onto diapers during manufacture or handling, it is unlikely that significant numbers of organisms would remain viable and be able to affect the normal skin flora. Since no increase in cfu/g occurred with time, these data also confirm the inability of common skin and fecal flora to utilize diaper materials as substrates.

The organisms which are naturally present in cellulose form the bioburden of diapers. Comparison of the survival and growth of the bioburden organisms of cloth, disposable and AGM diapers stored under warm, moist conditions which favor growth confirmed that even the resistant spore-forming populations of non-pathogens naturally present [10] did not increase in numbers. Together with the results from the persistence study, these data indicate that the diaper materials do not contribute organisms to the skin flora.

Collectively, the studies presented demonstrate that there are no major observable differences in the three diaper types with respect to growth and survival of representative skin and fecal microorganisms under the conditions tested. The single exception was that some organisms survived longer on cloth diapers than on disposable or AGM diapers. It is likely, as reported previously [7,13], that the containment of nutrients found in urine and feces and increased skin surface moisture contribute to the higher numbers of microorganisms associated with diapered area skin relative to undiapered skin. Additional in vitro and clinical studies have demonstrated that the numbers and types of skin flora are similar for children wearing cloth, disposable and AGM diapers [9].

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