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Investigation by Improved Syringe Method of Effect of Tampons on Production In Vitro of Toxic Shock Syndrome Toxin 1 by *Staphylococcus aureus*

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Twenty-seven types of commercial tampons from five manufacturers were tested in a sealed-syringe method to determine their effect on the growth of *Staphylococcus aureus* and the production of toxic shock syndrome toxin 1. In this improvement of the syringe method, the available air is limited to that which is contained within the sealed syringe containing the tampon. The culture medium was buffered, and blood and CO_2 were included in the incubation to better simulate the vaginal environment during menstruation. Variables of tampon weight, composition, air volume, and absorbency were examined for their effect on the production of toxic shock syndrome toxin 1. Generally, with the exception of brand E, toxin production in the presence of tampons was equal to or lower than that in a sealed control syringe containing air but no tampon.

Toxic shock syndrome (TSS), a multisystem disease associated with some strains of *Staphylococcus aureus* (16, 18), has been epidemiologically associated with the use of tampons by women during their menstrual period (8, 12, 16). An exoprotein, TSS toxin 1 (TSST-1), produced by the staphylococci isolated from patients with TSS has been accepted as an important cause of the disease (2, 3, 15). Several in vitro methods have been devised to try to understand what role, if any, tampons might play in the production of TSST-1 (7, 9–11, 14, 17). One of these methods, the syringe method (7), indicated that the composition of the tampons and treatments they receive might have an effect on the production of TSST-1.

One purpose of this study was to better simulate the vaginal environment during menstruation by limiting the available air to that present in the tampon, buffering the culture medium, and adding CO_2 and blood to the medium.

Another purpose was to better assess the reproducibility of the method by running five replicates of each sample on five separate days for each of two different *S. aureus* strains. This was more replication and randomness than has heretofore been reported.

MATERIALS AND METHODS

Staphylococcal strains. Two *S. aureus* strains, FRI-1169 and MN8, isolated from the vaginas of TSS patients, were used.

Tampons. Twenty-seven different sizes and types of tampons from five manufacturers were included in this study. All tampons were purchased commercially in July 1988, except for brand E, which was previously withdrawn from the market.

Culture medium. The medium consisted of brain heart infusion broth (Difco Laboratories, Detroit, Mich.) with the addition of 0.025 M phosphate buffer (pH 7.4). A volume of 10 parts of defibrinated rabbit blood (Remel, Lenexa, Kans.) was added to 90 parts of the medium. Petri dishes for enumerating CFU contained plate count agar (Difco).

Sample preparation. The tampons were removed from their wrappers and/or applicators. Strings were cut off the tampons, and the weights, lengths, and diameters were measured. Tampons were sterilized by being placed in 30-cm^3 disposable syringes (with plungers removed) (Becton Dickinson and Co., Rutherford, N.J.) which were then covered with aluminum foil and autoclaved. The rubber septa were removed from the syringe plungers and sterilized separately.

Assay for bacterial growth and TSST-1 production. The appropriate *S. aureus* strain was inoculated into 10 ml of brain heart infusion broth in a screw-cap culture tube and incubated at 37°C for 20 to 22 h. Separately, fresh medium (270 ml) containing 0.001% antifoam B emulsion (Sigma Chemical Co., St. Louis, Mo.) was sparged with 5% CO_2-95% air (Matheson Scientific, Inc., Rutherford, N.J.) to give a delivery volume of 33 cm^3 of gas per ml of medium. Thirty milliliters of rabbit blood was added to the sparged medium, which was then inoculated with 0.3 ml of the culture to give a count of ~10^6 CFU/ml (determined by plate count).

To each sterilized tampon in a syringe, 3 ml of inoculated medium was added per g of tampon. All syringes were then sealed by placing sterilized septa inside them at a precalculated volume, i.e., to include only the air volume from the tampon (see Calculations section and Table 1). Control syringes (no tampon) contained 10 ml of inoculated medium and 4 cm^3 of headspace. This 4 cm^3 of headspace was equivalent to the median air volume of the tampons used in this study. The syringes were incubated at 37°C for 22 h. Culture fluids were expressed by inserting the plunger and forcing it down on the inoculated tampon. Expressed culture fluids were measured for pH, and 0.1 ml was serially diluted with 0.1% peptone and surface plated on plate count agar plates to determine the bacterial population. The remainder of the expressed fluids was centrifuged (2,000 × *g*, 30 min) to remove bacterial cells. The concentration of TSST-1 in the supernatant fluid was determined serologically by a double-antibody sandwich enzyme-linked immunosorbent assay method (5).

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TABLE 1. Physical data for tampons[a]

Tampon	Fiber	Wt (g)	Syngyna absorbency[b] (g)	Air vol (cm^3)	Inoculum vol (ml)	Stopper vol (cm^3)
Brand A, deodorant						
Slender	Rayon	1.94	6.6	2.96	5.81	10.1
Regular	Rayon	2.65	8.6	4.28	7.94	14.0
Super	Rayon	2.92	10.1	5.52	8.75	16.2
Super Plus	Rayon	3.77	11.3	5.21	11.32	19.0
Brand A, nondeodorant						
Slender	Rayon	1.95	6.8	2.98	5.85	10.1
Regular	Rayon	2.62	8.7	4.24	7.84	13.8
Super	Rayon	2.94	10.0	5.43	8.83	16.2
Super Plus	Rayon	3.76	11.6	5.12	11.27	18.9
Brand B, cardboard applicator						
Junior	Rayon	1.72	5.0	1.89	5.15	8.2
Slender	Rayon	1.98	7.0	2.84	5.93	10.1
Regular	Cotton	2.37	7.0	4.11	7.11	12.9
Super	Rayon-cotton	3.05	9.7	5.07	9.15	16.2
Super Plus	Rayon-modified cellulose	3.48	13.0	4.45	10.44	17.2
Brand B, plastic applicator						
Regular	Rayon	1.97	6.9	2.78	5.90	10.0
Super	Rayon	2.69	9.4	3.89	8.06	13.7
	Rayon-modified cellulose	2.54	9.8	3.67	7.63	13.0
Super Plus	Rayon	3.57	12.6	4.46	10.68	17.5
	Rayon-modified cellulose	3.38	13.6	3.99	10.15	16.4
Brand C						
Slender	Rayon-cotton	2.04	7.3	2.89	6.10	10.3
Regular	Rayon-cotton	2.98	11.1	4.48	8.95	15.4
Super	Rayon-cotton	3.92	14.3	5.80	11.75	20.2
Super Plus	Rayon-cotton	4.74	16.6	7.89	14.20	25.2
Brand D, plastic applicator						
Regular	Rayon-cotton	2.66	7.8	4.36	7.97	14.1
Super	Rayon-cotton	3.96	11.2	9.27	11.89	23.8
Brand D, stick						
Regular	Rayon-cotton	3.25	7.4	2.45	9.74	14.3
Super	Rayon-cotton	4.36	9.7	3.07	13.09	19.0
Brand E, Super	Foam-carboxymethyl cellulose	3.20	18.5	8.42	9.60	20.7
Control				4.00	10.00	14.0

[a] Each value represents the average for the 10 tampons used in the study.
[b] In vitro absorbency as determined by the Syngyna absorbency method (23).

Five replicate assays per sample per strain were conducted on separate days. A total of 270 datum points (5 replicates × 27 samples × 2 strains) were produced in this study.

Calculations. Septum placement was designed to include only the amount of air introduced by each tampon. To determine where the septum should be placed in the syringe after the tampon had been inoculated, the tampons were treated as right cylinders for the purpose of calculations. The fiber volume (tampon weight/fiber density), the air volume in the tampon (π diameter^2/4 × length − tampon weight/fiber density), and the inoculum volume (3 × tampon weight) were added together to determine septum placement. Table 1 shows the combined tampon measurements used in this study.

Enzyme-linked immunosorbent assay analysis. A double-antibody sandwich enzyme-linked immunosorbent assay method with a detection limit of 0.625 ng/ml was used to assay for TSST-1 (5). A 96-well microdilution plate (Nunc, Roskilde, Denmark) was coated the day before the assay with rabbit immunoglobulin G specific for highly purified TSST-1 (>95% purity; Toxin Technology, Inc., Madison, Wis.). One hundred microliters of the immunoglobulin G solution (10 μg/ml in 0.01 M carbonate buffer, pH 9.6) was added to each well, and the plate was incubated at room temperature overnight. The next day, the plate was rinsed eight times with 0.01 M phosphate-buffered saline with 0.1% Tween 20 (PBS-TWN) to remove unbound antibodies. Serial dilutions of a partially purified preparation (>50% purity) of TSST-1 (Toxin Technology) in concentrations ranging from 0.625 to 10.0 ng/ml were made in PBS-TWN to generate the standard curve. The toxin preparation had been standardized against the highly purified TSST-1 by the manufacturer. Normal rabbit serum (final concentration, 5%) was routinely added to test samples to eliminate possible protein A interference (5). The toxin standards and blanks were treated similarly. Test standards and samples (100 μl) were added to each well and incubated for 2 h at room temperature. The

TABLE 2. Growth of *S. aureus* MN8 and FRI-1169 and production of TSST-1 when incubated with tampons[a]

Tampon	Fiber	MN8			FRI-1169		
		pH of recovered culture fluid	CFU/ml (10^9)	TSST-1 (ng/ml ± SD)	pH of recovered culture fluid	CFU/ml (10^9)	TSST-1 (ng/ml ± SD)
Brand A, deodorant							
Slender	Rayon	6.24	2.5	6.4 ± 2.3[b]	6.50	1.1	0.9 ± 0.2
Regular	Rayon	6.40	0.9	9.5 ± 6.4	6.43	0.3	1.2 ± 1.1
Super	Rayon	6.42	0.6	8.8 ± 4.9	6.42	0.2	0.8 ± 0.2
Super Plus	Rayon	6.42	1.2	8.4 ± 5.1	6.42	0.3	1.2 ± 1.0
Brand A, nondeodorant							
Slender	Rayon	6.42	2.2	21.1 ± 12.0	6.46	1.1	5.2 ± 3.6
Regular	Rayon	6.40	1.7	15.3 ± 11.3	6.43	0.6	2.6 ± 1.6
Super	Rayon	6.42	3.0	16.0 ± 7.6	6.44	0.9	4.2 ± 4.2
Super Plus	Rayon	6.39	1.6	29.7 ± 17.2	6.41	0.8	10.4 ± 8.0
Brand B, cardboard applicator							
Junior	Rayon	5.97	4.0	254.0 ± 128.7	5.93	1.6	139.4 ± 93.0
Slender	Rayon	6.12	6.3	178.0 ± 99.2	6.10	2.8	148.9 ± 133.6
Regular	Cotton	6.26	1.3	84.0 ± 17.7	6.29	0.6	33.7 ± 27.0
Super	Rayon-cotton	6.21	0.7	85.6 ± 27.5	6.23	0.6	22.7 ± 20.1
Super Plus	Rayon-modified cellulose	6.19	7.4	103.6 ± 48.4	6.15	4.7	82.0 ± 29.8
Brand B, plastic applicator							
Regular	Rayon	6.18	3.4	199.1 ± 93.1	6.18	2.2	151.8 ± 35.2
Super	Rayon	6.17	5.6	189.6 ± 95.1	6.14	4.6	106.2 ± 66.4
	Rayon-modified cellulose	6.22	11.0	196.0 ± 83.4[c]	6.17	7.0	146.7 ± 83.1
Super Plus	Rayon	6.19	8.1	119.3 ± 48.3	6.17	1.4	100.8 ± 54.9
	Rayon-modified cellulose	6.24	15.0	107.8 ± 56.7	6.22	8.2	83.7 ± 45.7
Brand C							
Slender	Rayon-cotton	6.31	1.6	8.2 ± 6.2	6.36	2.4	2.6 ± 1.1[c]
Regular	Rayon-cotton	6.31	3.0	4.8 ± 2.1	6.37	5.6	1.8 ± 0.8[c]
Super	Rayon-cotton	6.31	4.7	13.6 ± 8.5	6.32	1.3	3.0 ± 0.7
Super Plus	Rayon-cotton	6.34	4.9	37.9 ± 16.0	6.34	3.2	69.3 ± 64.6
Brand D, plastic applicator							
Regular	Rayon-cotton	6.40	0.4	71.7 ± 9.8	6.40	0.2	23.0 ± 5.4
Super	Rayon-cotton	6.39	0.7	57.6 ± 19.7	6.43	0.2	39.5 ± 21.0
Brand D, stick							
Regular	Rayon-cotton	6.41	0.2	82.2 ± 24.8	6.37	0.3	25.5 ± 14.9
Super	Rayon-cotton	6.39	0.4	73.9 ± 23.8	6.36	0.1	17.1 ± 9.9
Brand E, Super	Foam-carboxymethyl cellulose	6.41	3.0	1,035.7 ± 468.9	6.36	0.7	819.3 ± 414.1
Control		6.43	2.5	180.9 ± 105.1	6.31	2.5	142.4 ± 65.6

[a] Five replicates for each tampon per strain except where noted otherwise.
[b] Average of three replicates.
[c] Average of four replicates.

plate was again rinsed eight times with PBS-TWN. Fifty microliters of horseradish peroxidase conjugated to anti-TSST-1 immunoglobulin G (Toxin Technology) was added to each well, and the plate was then incubated for 30 min at 37°C. The plate was rinsed with PBS-TWN, and 100 μl of the substrate (1.2 nM H_2O_2 plus 0.6 nM 2,2′-azino-di-3-ethylbenzthiazoline sulfonic acid [ABTS; Sigma]) in 0.05 M citric acid buffer, pH 4.0, was added. The A_{410} was read with a model 700 microplate reader (Cambridge Technology, Inc., Watertown, Mass.). All tests were run in triplicate.

Analysis of data. Data were analyzed by using an analysis of variance test available as a Statistical Analysis System program.

RESULTS

The final pH of the recovered culture fluid per tampon, the CFU, and the amount of TSST-1 recovered per milliliter for both strains MN8 and FRI-1169 are given in Table 2. In general, with the exception of brand E, tampons either inhibited or had no effect on TSST-1 production. Differences in TSST-1 production between strains were analyzed by using analysis of variance and were shown to be significant ($P < 0.05$). Although higher TSST-1 levels were produced by strain MN8, the relative rank orders were similar for both strains.

The reproducibility and precision of the enzyme-linked immunosorbent assay method used was tested by retaining

TABLE 3. Rank order of studied variables by tampon brand[a]

Tampon	Fiber	Wt	Air vol	Syngyna absorbency[b]	TSST-1 production by strain:	
					MN8	FRI-1169
Brand A, deodorant						
Slender	Rayon	2	6	2	2	2
Regular	Rayon	10	15	10	6	4
Super	Rayon	13	24	17	5	1
Super Plus	Rayon	23	22	20	4	3
Brand A, nondeodorant						
Slender	Rayon	3	7	3	10	10
Regular	Rayon	9	14	11	8	7
Super	Rayon	14	23	16	9	9
Super Plus	Rayon	22	21	21	11	11
Brand B, cardboard applicator						
Junior	Rayon	1	1	1	27	23
Slender Regular	Rayon	5	4	5.5	22	26
Regular	Cotton	7	13	5.5	17	16
Super	Rayon-cotton	16	20	13.5	18	13
Super Plus	Rayon-modified cellulose	20	17	23	19	19
Brand B, plastic applicator						
Regular	Rayon	4	3	4	26	27
Super	Rayon	12	10	12	24	22
	Rayon-modified cellulose	8	9	15	25	25
Super Plus	Rayon	21	18	22	21	21
	Rayon-modified cellulose	19	11	24	20	20
Brand C						
Slender	Rayon-cotton	6	5	7	3	6
Regular	Rayon-cotton	15	19	18	1	5
Super	Rayon-cotton	24	25	25	7	8
Super Plus	Rayon-cotton	27	26	26	12	18
Brand D, plastic applicator						
Regular	Rayon-cotton	11	16	9	14	14
Super	Rayon-cotton	25	28	19	13	17
Brand D, stick						
Regular	Rayon-cotton	18	2	8	16	15
Super	Rayon-cotton	26	8	13.5	15	12
Brand E, Super	Foam-carboxymethyl cellulose	17	27	27	28	28
Control			12		23	24

[a] Each variable ranked on a scale of 1 to 28, with 1 representing the lowest value and 28 representing the highest value.
[b] In vitro absorbency as determined by the Syngyna absorbency method (23).

culture supernatants of two different tampons and a control sample and assaying for TSST-1 four times over a 4-week period. Results of these assays were consistent with those obtained on the day the culture fluid was harvested and initially assayed (results not shown).

DISCUSSION

TSS was first described in 1978 (18). It is important to note that in vitro and animal models have not yet been able to explain the etiology of, or to fully replicate, this disease. It is acknowledged that in vivo microbe-host factors, such as host immune status and effects of growth conditions, can at best only be approximated in an in vitro model (19).

This study, however, was an in vitro attempt to understand how variables in commercial tampons may or may not affect TSST-1 production. The syringe method described by Lee et al. (7) approximates the in vivo situation to the extent that the expansion of the tampon is restricted as it would be in the vagina. In a further attempt to simulate the vaginal environment and to improve its precision, the method was modified by limiting the O_2 to that which is contained within the tampon and sparging the medium with 5% CO_2 to approximate carbon dioxide tension in the vagina (21). Additionally, the inoculating medium was buffered to a neutral pH to simulate the buffering capacity and pH of menstrual fluid (1, 4). After incubation, the final pH values were mostly in the neutral range (Table 2). When buffer was not added, the pH dropped to below 6 (data not shown). The vaginal pH during menstruation was reported to be near neutral (22).

In this system, the presence of tampons either inhibited or had no effect on TSST-1 production compared with that of the control (only brand E Super tampons resulted in significantly higher TSST-1 levels). This corresponded with findings by Schlievert et al. (14) but not with reports of other investigators (7, 9, 10, 17).

It was anticipated that the effects of weight, air content, and absorbency on TSST-1 levels might be discernible, particularly among those brands which maintained a constant fiber composition for all sizes. These data, which are rank ordered in Table 3, do not, however, indicate any relationship between the amount of TSST-1 produced and any of the variables mentioned above, except for brand E Super, which had the second highest air volume and the highest Syngyna absorbency and TSST-1 ranking.

The amount of air present was of particular interest since Todd et al. (20), Kass et al. (6), and Schlievert and Blomster (13) showed that more TSST-1 was produced under aerobic than anaerobic conditions. Since the available air was limited to that which is contained within the tampon, it allowed focus to be placed on the role of the air volume within the tampon in the production of TSST-1. It was hypothesized that production of TSST-1 would increase as air volumes increased; however, this did not appear to be true. Looking at homologous series in which increasing weight was generally accompanied by increasing air volume, there was no concurrent increase in TSST-1. Brand B Junior, the tampon calculated to contain the smallest air volume, fell in the upper range of TSST-1 recovered, whereas brand D Super with the largest air volume afforded the production of less TSST-1.

In the syringe method of Lee et al. (7), which included 10% blood in the medium and in which the amounts of air and CO_2 available to the syringes were not controlled, less TSST-1 was recovered in the control syringes than in the syringes containing tampons. In contrast, the stationary control cultures in this study, in which the headspace was limited to 4 cm^3 of air, produced some of the highest TSST-1 values obtained. These results illustrate the importance of regulating the available amounts of air and CO_2 in in vitro methods. The results of this study also indicate that relatively little air is required for TSST-1 production.

In contrast to the study by Lee et al. (7), this study indicates an inhibition of toxin production by most tampons in comparison with the control syringes. In some cases, this result appears to be related to the compositions of the tampons. It appears, with the exception of several styles of brand B, that rayon, cotton, and blends thereof inhibit production of TSST-1 in comparison with the control containing no tampon. Those tampons composed of modified rayons and foam-carboxymethyl cellulose chips afford TSST-1 values equal to or higher than that of the control culture.

Less TSST-1 was produced in brand A deodorant tampons than in the nondeodorant counterparts. These results implied an inhibitory effect of the deodorant, as suggested by Lee et al. (7) and Robbins et al. (11).

To eliminate the variables of tampon configuration and degree of compactness and to determine whether they contributed to the production of TSST-1, selected tampons having different configurations (brand B Slender and brands A, B, and C Regular) were mechanically shredded on an analytical mill (Arthur H. Thomas Co., Philadelphia, Pa.) to a particle size of approximately 0.081 cm. The shredded fibers were inoculated and incubated in the sealed-syringe method and were measured for bacterial growth and TSST-1 production along with their whole-tampon counterparts. The rank orders for production of TSST-1 were similar for the whole and shredded tampons (results not shown).

In conclusion, an improved reproducible method that attempts to simulate various aspects of the vaginal environment during menstruation has been reported. In this method, increases in absorbency, air volume, size, and configuration of the tampon did not correlate with increases in TSST-1 production, with the exception of brand E, and very little O_2 was needed to trigger production of TSST-1. It is also clear from this study that, with the exception of brand E, the level of TSST-1 produced in the control was equal to or higher than that produced in the presence of tampons.

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