Evidence for the Absence of *Staphylococcus aureus* in Land Applied Biosolids

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Staphylococcus aureus is an important human pathogen both within the hospital setting and as a community-acquired infection. Recently there has been concern that land applied biosolids may transmit *S. aureus*. However, no scientific data are available to document whether biosolids are a source of *S. aureus*. To determine if *S. aureus* is present in biosolids, we collected samples from 15 sites across the United States. Samples analyzed were as follows: 3 raw untreated sewage samples and 2 undigested primary sewage sludge samples; 23 different biosolid samples; and 27 aerosols obtained during biosolid land application (biosolid aerosols). Although *S. aureus* were detected in raw sewage samples, none were found in any of the treated biosolids nor in any biosolid aerosol samples. These results suggest that biosolids are not a likely source of *S. aureus* human exposure or infection.

Introduction

Biosolids are organic residues that result from the treatment of municipal sewage *(1)*. Thus the term biosolids implies treatment to defined levels, and biosolids are chemically and biologically different than raw sewage. The U.S. Environmental Protection Agency has established two categories of biosolids that can be land applied *(2)*. Class A biosolids have undergone sufficient treatment to reduce enteric pathogens to low enough levels that no special handling precautions are required by federal regulations. Class A biosolids meet the following criteria: <1000 FC g^{-1} by MPN, <3 *Salmonella* 4 g^{-1} total solids (TS), <1 plaque-forming viral unit (PFU) 4 g^{-1} TS, and <1 viable helminth 4 g^{-1} TS. Class B biosolids have been treated to reduce numbers of enteric pathogens but not to eliminate them completely. Class B biosolids should contain $<2\,000\,000$ fecal coliforms g^{-1} TS *(3)*. Federal regulations for the use of class B biosolids require measures to restrict public access and to limit livestock grazing for specified time periods after land application. This allows time for the natural die-off of pathogens in the soil.

S. aureus is the agent of a wide variety of human infections including skin and wound infections, food poisoning, septicemia, toxic shock syndrome, endocarditis, osteomyelitis, pneumonia, and meningitis *(4)*. The major habitats of *S. aureus* include the nares, skin, and to a lesser extent, the gastrointestinal tract and genital tract of warm-blooded animals.

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Although there is no scientific documentation of *S. aureus* transmittal from wastewater or biosolids, recently there have been suggestions that this potential exists from land applied biosolids *(5)*. A related study suggested that approximately 25% of 48 individuals who lived near land application sites and who had complained of chemical irritation had evidence of *S. aureus* infections *(6)*. However, the authors of this study admit that no control group was included in the investigation to assess background levels of *S. aureus* infections in the population. In addition, no direct linkage of *S. aureus* to land applied biosolids was documented. Nevertheless, this study has raised serious questions regarding the possible acquisition of *S. aureus* infections from land applied biosolids. The objective of this present study was to examine biosolids and related aerosols for the presence of *S. aureus*. Therefore, we evaluated the occurrence of *S. aureus* in sewage, class A and class B biosolids, and aerosols obtained during land application of biosolids.

Experimental Methods

Sewage and Biosolid Sampling. Samples of both sewage and biosolids were collected from several different locations nationally. Biosolids representing all major treatment systems were included in this study from 15 different locations ranging from the Southwest United States to the East coast. All of the sites in this study were full-scale plants; no pilot plants were included. The average daily flow of each plant is shown in Table 1. Raw sewage and untreated primary sewage sludge samples were collected in sterile Nalgene bottles and transported to the laboratory on ice. Biosolid samples were collected in sterile plastic bottles or Whirl-Pak bags (Nasco, Modesto, CA) at the site of production and transported or mailed overnight to the laboratory on ice. Samples were assayed for *S. aureus* on the day of receipt. Information regarding the treatment utilized to generate sewage and biosolid samples was obtained from each wastewater treatment plant (Table 1).

Aerosol Sampling. All samples were collected during land application of biosolids. Air sampling was performed using a Vac-U-Go noncompositing pump (SKC, Eighty four, PA) at a pump rate of 12.5 L min^{-1}. The SKC biosampler included a sterile impinger in which 50% of the particles that are allowed to pass are 0.3−5.0 μm in diameter. Air samples were collected in 0.1% peptone water (Difco, Sparks, MD). Air samples were obtained from a height of 1.5 m above the ground and transported to the laboratory on ice in 50-mL sterile conical centrifuge tubes. All samples were analyzed for *S.* aureus within 24 h of collection. Four different sites in the Southwestern United States were included. Multiple samples were sometimes collected from the same site on the same day. A variety of environmental conditions including temperature, relative humidity, and wind speed were encountered during the study (Table 2).

Background air samples were obtained 91.4 m upwind of biosolid application sites prior to the application of the biosolids. At Southwest sites 1−3, biosolids were applied utilizing a Betterbuilt spray tanker pulled by a tractor. These biosolids were sprayed 1.8 m into the air, and aerosol samples were collected during the spraying procedure. Southwest site 4 biosolids were loaded into the field via a dump truck. A front-end loader then transferred the biosolids into a hopper that was used to spread the product over the field. At site 4, aerosol samples were obtained downwind of application sites during times when biosolids were being unloaded from the delivery truck, loaded into the hopper, and when being applied to the field. Southwest sites 1−3 aerosol samples

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TABLE 1. *Staphylococcus aureus* in Sewage and Biosolid Samples

date	site and sample	treatment	output of plant ($m^3\ d^{-1}$)	*S. aureus* ($100\ g^{-1}$)
		Raw Sewage		
04/05/02	Southwest site 1	none	94 625	negative
05/30/02	Southwest site 1	none		positive
07/29/02	East Coast site 1	none	14 383	<30
		Undigested Primary Sewage Sludge		
05/29/02	Southwest site 1	thickened	94 625	positive
06/20/02	Southwest site 1	thickened		150
		Biosolids—Class B		
02/22/02	Southwest site 1	anaerobic mesophilic digestion	94 625	negative
04/05/02	Southwest site 1	anaerobic mesophilic digestion		negative
05/03/02	Southwest site 1	anaerobic mesophilic digestion		negative
06/19/02	Southwest site 1	anaerobic mesophilic digestion		<30
08/08/02	Southwest site 2	anaerobic mesophilic digestion	635 880	<30
07/29/02	East Coast site 1	anaerobic mesophilic digestion	14 383	<30
08/14/02	East Coast site 2	aerobic mesophilic digestion	17 411	<30
08/12/02	East Coast site 3	aerobic mesophilic digestion, lime	7570	<30
08/12/02	East Coast site 3	aerobic mesophilic digestion, lime		<30
08/12/02	East Coast site 4	aerobic mesophilic digestion, lime	11 355	<30
08/12/02	East Coast site 4	aerobic mesophilic digestion, lime		<30
08/12/02	East Coast site 5	aerobic mesophilic digestion, lime	75 700	<30
08/12/02	East Coast site 5	aerobic mesophilic digestion, lime		<30
08/14/02	East Coast site 2	aerobic mesophilic digestion, lime	17 411	<30
08/22/02	East Coast site 6	aerobic mesophilic digestion, lime	4542	<30
07/29/02	East Coast site 1	anaerobic mesophilic digestion, lime	14 383	<30
		Biosolids—Class A		
07/29/02	East Coast site 7	aerobic thermophilic digestion	4920	<30
08/09/02	East Coast site 8	anaerobic thermophilic digestion	321 725	<30
08/13/02	East Coast site 9	heat dried pellets made after anaerobic mesophilic digestion	757 000	<30
08/12/02	East Coast site 10	heat dried pellets made from undigested sewage	132 475	<30
08/13/02	East Coast site 11	heat dried pellets following anaerobic mesophilic digestion	681 300	<30
08/16/02	East Coast site 12	heat dried pellets from mixture of undigested thickened primary and thickened waste activated sludge	333 080	<30
08/09/02	East Coast site 13	composted aerobic pile	45 450	<30

TABLE 2. Environmental Parameters Associated with Biosolid Aerosol Sample Collection

date	site	temp (°C)	% rel humidity	wind speed ($m\ s^{-1}$)
02/15/02	Southwest site 1	16.6	12.3	1.2
02/22/02	Southwest site 1	22.3	4	1.1
04/05/02	Southwest site 2	24.8	6.5	0.5
06/19/02	Southwest site 3	26	13	1.5
08/06/02	Southwest site 4	22.4	38	2.6
08/07/02	Southwest site 4	20.6	50	2.3
08/08/02	Southwest site 4	28.1	15	3.6

were obtained 2 m from the application site, while at Southwest site 4, aerosol samples were collected at distances of 2−32 m from locations where biosolids were being handled (Table 3).

Presence/Absence of *S. aureus*. Initial samples were evaluated for *S. aureus* on a presence/absence basis. Ten-fold dilutions of biosolid or sewage samples were prepared in sterile physiological (0.85%) saline and directly plated unto Mannitol Salt Agar (MSA, Difco) and Baird Parker Medium (BP, Difco).

Enumeration of *S. aureus*. *S. aureus* was enumerated in most samples utilizing the Most Probable Number (MPN) method performed with M Staphylococcus enrichment broth (Difco). All tubes were incubated for 24 h at 35 °C. Tubes demonstrating turbidity were subcultured after 24 h, while nonturbid tubes were subcultured after another 24 h of incubation. Subsamples from each tube were subcultured to MSA and BP media.

Identification of *S. aureus*. MSA and BP plates were incubated 48 h at 35 °C. Colonies typical of *S. aureus* (*7*) were subcultured to 5% Sheep Blood Agar (BA).

BA plates were incubated 24−48 h at 35 °C and inspected for colonies typical of *S. aureus*. Colonies typical of *S. aureus* are beta-hemolytic, smooth, entire, slightly raised, and translucent. The colonies are usually cream-yellow to orange (*7*). Typical isolates were tested utilizing catalase production, microscopic morphology, and tube coagulase production. Isolates that were gram-positive cocci, catalase positive, coagulase positive, clumping factor positive (slide coagulase test positive), and resistant to polymyxin B were identified as *S. aureus* (*7*).

Tube Coagulase Test. Catalase positive, gram-positive cocci were tested by the tube coagulase test utilizing dehydrated rabbit plasma containing EDTA (Difco). Large isolated colonies were placed into 0.5 mL of reconstituted plasma and incubated at 35 °C. Each tube was inspected for free coagulase production after 4 and 24 h incubation (*7*). Any degree of clotting constituted a positive test.

Slide Coagulase Test. Isolates that were positive by the tube coagulase method were subjected to the slide coagulase test as *S. schleiferi* subs. *coagulans*, *S. lutae*, *S. intermedius*, *S. delphini*, and *S. hyicus* may be tube coagulase positive. The slide coagulase test was performed by making a heavy homogeneous suspension of growth in distilled water on a glass slide and gently mixing in one drop of plasma. Clumping within 10 s constituted a positive test.

Polymixin B Resistance. This test was used to distinguish *S. intermedius* from *S. aureus*. A 300-U polymixin B disk (BBL, Cockeyville, MD) was utilized. A suspension of the isolate was made into sterile physiological saline equivalent to a 0.5 McFarland opacity standard (*7*). A lawn of the suspension was made using a sterile cotton-tipped swab unto BA. The disk was placed unto the agar, and the plate was incubated at 35 °C for 18−24 h. A zone of inhibition of ≤10 mm was defined as resistant (*7*).

Efficiency of MPN Recovery Method in Biosolids. An inoculum of *S. aureus* ATCC 25923 (American Type Culture Collection, Rockville, MD) was prepared by 18-h growth in

TABLE 3. Analysis of Bioaerosols for *Staphylococcus aureus*

date	site	sample	bioaerosol sample description	*S. aureus* (m^{-3})
			Background Aerosols	
02/08/02	Southwest site 1	1	91.4 m upwind of sites prior to land application of biosolids	<43[a]
02/15/02	Southwest site 1	1	91.4 m upwind of sites prior to land application of biosolids	<39
02/22/02	Southwest site 1	1	91.4 m upwind of sites prior to land application of biosolids	<80
02/22/02	Southwest site 1	2	91.4 m upwind of sites prior to land application of biosolids	<101
02/22/02	Southwest site 1	3	91.4 m upwind of sites prior to land application of biosolids	<80
04/05/02	Southwest site 2	1	91.4 m upwind of sites prior to land application of biosolids	<63
04/05/02	Southwest site 2	2	91.4 m upwind of sites prior to land application of biosolids	<63
06/19/02	Southwest site 3	1	91.4 m upwind of sites prior to land application of biosolids	<19
06/19/02	Southwest site 3	2	91.4 m upwind of sites prior to land application of biosolids	<19
08/07/02	Southwest site 4	1	91.4 m upwind of sites prior to land application of biosolids	<28
			Biosolid Aerosols	
02/15/02	Southwest site 1	1	2 m downwind of liquid spray biosolids	<155
02/15/02	Southwest site 1	2	2 m downwind of liquid spray biosolids	<149
02/15/02	Southwest site 1	3	2 m downwind of liquid spray biosolids	<147
02/15/02	Southwest site 1	4	2 m downwind of liquid spray biosolids	<145
02/22/02	Southwest site 1	1	2 m downwind of liquid spray biosolids	<106
04/05/02	Southwest site 2	1	2 m downwind of liquid spray biosolids	<23
04/05/02	Southwest site 2	2	2 m downwind of liquid spray biosolids	<23
04/05/02	Southwest site 2	3	2 m downwind of liquid spray biosolids	<23
04/05/02	Southwest site 2	4	2 m downwind of liquid spray biosolids	<23
06/19/02	Southwest site 3	1	2 m downwind of liquid spray biosolids	<19
06/19/02	Southwest site 3	2	2 m downwind of liquid spray biosolids	<20
06/19/02	Southwest site 3	3	2 m downwind of liquid spray biosolids	<29
06/19/02	Southwest site 3	4	2 m downwind of liquid spray biosolids	<20
08/06/02	Southwest site 4	1	23−32 m downwind of hopper applying biosolids	<18
08/06/02	Southwest site 4	2	20−29 m downwind of hopper applying biosolids	<18
08/06/02	Southwest site 4	3	19−26 m downwind of hopper applying biosolids	<55
08/06/02	Southwest site 4	4	16−23 m downwind of hopper applying biosolids	<55
08/06/02	Southwest site 4	5	5−19 m downwind of hopper applying biosolids	<22
08/06/02	Southwest site 4	6	2−16 m downwind of hopper applying biosolids	<22
08/07/02	Southwest site 4	7	11 m downwind of truck unloading biosolids	<28
08/07/02	Southwest site 4	8	8 m downwind of truck unloading biosolids	<28
08/07/02	Southwest site 4	9	11 m upwind of truck unloading biosolids	<28
08/07/02	Southwest site 4	10	8 m upwind of truck unloading biosolids	<28
08/08/02	Southwest site 4	11	15 m downwind of loading biosolids into hopper	<18
08/08/02	Southwest site 4	12	15 m downwind of loading biosolids into hopper	<28
08/08/02	Southwest site 4	13	2 m downwind of loading biosolids into hopper	<18
08/08/02	Southwest site 4	14	2 m downwind of loading biosolids into hopper	<28

[a] Indicates limit of detection sensitivity.

Tryptic Soy Broth (Difco) at 35 °C. An aliquot of class B biosolid sample from Southwest site 1, obtained 06/19/02, was seeded with 5.3×10^2 CFU *S. aureus* g^{-1}. The MPN procedure was utilized on the seeded biosolid sample and efficiency was determined by comparing number of *S. aureus* recovered g^{-1} to the known number seeded g^{-1} in the sample.

Quality Control of Media and Reagents. The selectivity of the MSA and BP media were confirmed using *E. coli* ATCC 25922 and *S. aureus* ATCC 25923. *S. aureus* ATCC 25923 also served as a positive control for the tube and slide coagulase tests, while uninoculated plasma served as negative controls. The potency of the 300-U polymixin disks were confirmed using *Pseudomonas aeruginosa* ATCC 27853.

Results

When 5.3×10^2 CFU *S. aureus* g^{-1} was seeded into a class B biosolid sample, previously negative for *S. aureus*, 4.6×10^1 CFU g^{-1} was detected via the MPN method used in this study. This indicates a recovery efficiency of 8.7%.

S. aureus was isolated in one of the three raw untreated sewage samples and two undigested primary sewage sludge samples (Table 1). However, no *S. aureus* (<30 100 g^{-1}) were detected in any of the seven class A or 16 class B biosolids tested (Table 1).

No *S. aureus* were detected in any background aerosol or biosolid aerosol sample (Table 3). Hence. *S. aureus* were not detected in air samples even when aerosol samples were obtained within 2 m of the site of land application and during the times that the biosolids were being applied either by spraying or by hopper utilization. The minimum detectable level of *S. aureus* varied depending upon the volume of air that was sampled.

Discussion

The MPN method of detection was utilized in this study rather than polymerase chain reaction (PCR) in order to enumerate only viable *S. aureus*. The efficiency of recovery of the MPN method when analyzing biosolids was estimated as 8.7%. Our MPN method compares favorably with a detection limit of approximately 10^7 *S. aureus* 100 mL^{-1} utilizing an ELISA method based on immunomagnetic beads for the detection of *S. aureus* thermostable nuclease *(8)*.

Analyses of sewage sludges or biosolid samples for *S. aureus* have been rare and frequently done without rigorous confirmation of the isolates. Dudley et al. *(9)* did not detect staphylococci in biosolid samples. In contrast, untreated lagooned sludge was reported to contain staphylococci. Although these authors performed a gram stain on each isolate, no other confirmatory tests were included. Hence, other genera of gram positive cocci may have been identified as staphylococci. In addition, no attempt to determine the presence of *S. aureus* was made.

Staphylococci have been reported in aerosols from wastewater plants using aeration treatments. Eikmann et al. *(10)* isolated approximately 250 aerosolized staphylococci m^{-3} at various sites throughout a wastewater treatment plant.

Typical colonies on mannitol salt agar strips were presumed to be staphylococci. However, no confirmation tests of any kind were performed. Hence, these estimates may be higher than the actual case. As with the previous investigators, *S. aureus* was not differentiated from other members of its genus.

Levels of 26−100 CFU/m^{-3} staphylococci were reported isolated from aerosols generated during the mechanical aeration of wastewater during the activated sludge process of wastewater effluent *(11)*. Many of these staphylococci (16−40%) were identified as *S. aureus* based on a positive tube coagulase reaction. However, these authors state that MSA was used for the isolation of staphylococci and that colonies grown on this agar were processed for the coagulase test using the Staphytect plus (Oxoid, Ogdensburg, NY). The authors do not indicate that microscopic morphology was determined for isolates identified as staphylococci. This step is important because bacterial species other than *S. aureus* are able to produce coagulase *(7)*. Hence, it is essential to be sure that the isolate tested is a gram-positive cocci before proceeding with a coagulase test. We found that many bacteria isolated on MSA, including those forming yellow colonies on the medium, are not members of the genus *Staphylococcus* (data not shown). Accepting that *S. aureus* was present in some of the aerosol samples examined by Brandi et al. *(11)*, this does not conflict with our findings. We detected *S. aureus* in samples of raw sewage and undigested primary sewage sludge. The presence of *S. aureus* in effluent treated by the activated sludge process is quite possible. However, we did not detect *S. aureus* in class A or class B biosolids after aerobic or anaerobic digestion, lime stabilization, heat-dry pelleting, and/or composting.

Staphylococci were also recovered from aerosols at several points at a wastewater treatment plant utilizing anaerobic digestion and covered biological oxidation lines in the treatment train *(12)*. *S. aureus* was detected in 0−60% of these samples. Most of the *S. aureus* were identified near primary sedimentation tanks, inside the chemical analysis laboratories, and near the exit of secondary effluent from secondary sedimentation tanks. However, the investigators did not determine how far *S. aureus* was carried in an aerosol from any one point within the treatment plant to another. This is not necessarily a weakness in the methods design as this would be very difficult to determine. Nevertheless, it does mean that small numbers of *S. aureus* detected at any one site in the treatment may have originated at another site in the plant further sway from the aerosol sampler. Also, aerosol samplers were placed at sites in the treatment plant along the route used by staff during the normal course of work. This being the case, it is possible that some of the staphylococci isolated originated from the workers walking by the samplers. It has been shown that staphylococci are dispersed in large numbers into the air when colonized individuals are moving *(13)*. This may be part of the reason that relatively high numbers of *S. aureus* were isolated from aerosol in the chemical analysis laboratory while technicians were working.

None of the background or biosolid aerosol samples in the present study were positive for *S. aureus*. To be classified as *S. aureus*, several confirmatory tests were employed in order to separate *S. aureus* from other species of tube coagulase positive species of staphylococci and other bacteria. It is important to include these confirmatory tests as *S. aureus* stands out as a medically important species.

These results suggest that biosolids are not a significant source of *S. aureus* human exposure or source of *S. aureus* infection in humans. Investigators should consider careful and rigorous confirmation steps for the identification of any bacterial pathogens in environmental samples as no medium is absolutely selective and differential. These confirmation steps are important because the identification of human pathogens in environmental samples (not only biosolids) often have far-reaching effects on regulatory policy and public perception.

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