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Combined Serotyping and Biotyping of *Serratia marcescens*

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The API (Analytab Products, Inc., New York, N.Y.) biotypes of 117 clinical isolates of *Serratia marcescens* were determined and fell into 13 different patterns. The O and H antigens were determined by tube agglutination, and 27 serotypes were identified. The biotype and serotype appeared to vary independently. Serotyping and biotyping combined divided these isolates into 56 different types. There was a problem interpreting the end points for inositol fermentation and urease production, which could affect reproducibility of API biotypes. Biotyping is a simple way of screening for possible nosocomial outbreaks of *S. marcescens*.

Serratia marcescens, once considered a harmless saprophyte, is being reported with increasing frequency as an agent of nosocomial infection (1, 2, 4, 13, 14). Most isolates are recovered from respiratory specimens and urine (11), but there are reports of *S. marcescens* septicemia (5), pneumonia (13), wound infection (11), and arthritis (6).

Detection of a hospital outbreak is often dependent on subdivision of isolates below the species level. Methods for subdividing bacteria have been based on serology, bacteriocin production and sensitivity, bacteriophage sensitivity, antibiogram, and biotype. Using antigens to 13 flagellar (H) antigens and 15 somatic (O) antigens, Edwards and Ewing (7) were able to serotype about 95% of cultures tested. Serotyping has been used successfully to study several nosocomial outbreaks involving *S. marcescens* (13, 16). Bacteriocin production (8) and bacteriocin sensitivity (9, 15) also have been used to differentiate strains of *S. marcescens*. Farmer (8) found that bacteriocin production was more stable than bacteriocin sensitivity, and he was able to type 91% of the strains tested. A bacteriophage typing scheme that divides *S. marcescens* into 71 bacteriophage types has been developed (10), with about 90% of the isolates being lysed by at least one phage.

Recently, Rennie and Duncan (12) reported that *Klebsiella* could be divided into 29 distinct biotypes using conventional media for biotype determination. The Analytab Products, Inc. (API; New York, N.Y.) 20E system for identification of *Enterobacteriaceae* is used routinely in many clinical laboratories and provides a "standardized" method for biotyping.

In this study we compared the biotypes and serotypes of clinical isolates of *S. marcescens* and assessed the feasibility of using the API biotype for epidemiological surveillance.

MATERIALS AND METHODS

***S. marcescens* isolates.** All of the strains were isolated over a 14-month period from specimens submitted to the clinical microbiology laboratory of Saint Francis Hospital, Hartford, Conn. They were identified as *S. marcescens* in the clinical laboratory by the Rb (Diagnostic Research, Corning, N.Y.) system for identification of *Enterobacteriaceae*. Each isolate was numbered consecutively and not decoded until completion of the biotyping so that isolates from the same patient could be compared without bias. Strains were grown in skim milk and frozen at −20 C. Working cultures were maintained on brain heart infusion agar (BBL) slants and transferred every 3 months.

Biotyping. The API micromethod was used for biotyping and included the following tests: *O*-nitrophenyl-β-D-galactosidase (ONPG), arginine dihydrolase (ARG), lysine (LDC) and ornithine decarboxylase (ODC), citrate utilization (CIT), H_2S production (H_2S), urease (URE), tyrosine deaminase (TDA), indole (IND) production, acetoin production (VP), gelatinase (GEL), and fermentation of glucose (GLU), mannitol (MAN), inositol (INO), sorbitol (SOR), rhamnose (RHAM), sucrose (SAC), melibiose (MEL), amygdalin (AMY), and arabinose (ARA).

The strips were inoculated according to the manufacturer's instructions. A single colony was picked with a sterile wooden applicator stick and suspended in 5 ml of sterile saline (0.85%). A Monstr-pette (Chase Instruments) was used to inoculate the wells. In addition to overlaying the ARG, LDC, ODC, and URE cupules as recommended by the manufacturer, we also overlaid the arabinose cupule with heavy-grade mineral oil. *S. marcescens* may oxidize this sugar, producing enough acid to change the indicator in the entire cupule and resulting in erroneous identification.

The strips were incubated for 18 h in the plastic

container supplied by the manufacturer, in which 5 ml of water was placed to maintain humidity. Results of the 20 biochemical tests and the oxidase (OX) test were converted to a 7-digit numerical biotype as directed by the manufacturer. These 21 biochemical reactions are divided into seven groups of three in their order on the strip, with oxidase being last. In each group of three, a positive reaction in place 1 is given a value of 1; place 2, a value of 2; and, in place 3, a value of 4. Negative reactions are given a zero. Each group of three is totaled to give a single digit. The series of 7 digits is the numerical biotype. For example: an organism producing the following biochemical reactions has an API biotype of 5 307 721: ONGP+, ARG−, LDC+; ODC+, CIT+, H_2S−; URE−, TDA−, IND−; VP+, GEL+, GLU+; MAN+, INO+, SOR+; RHAM−, SAC+, MEL−; AMY+, ARA−, OX−. The API computer service was used.

Each isolate was tested independently by two of the authors (S.J.R. and M.C.). Unusual biotypes were confirmed in conventional biochemical tests and, in some cases, were sent to the Connecticut State Health Department Laboratory for further confirmation.

Serotyping. Serotypes were determined by tube agglutination according to the method of Edwards and Ewing (7). Commercial antisera were used (Lee Laboratories, Grayson, Ga.). After dilution in saline, antisera were stored at 4 C for 1 week and then discarded, because after 1 week flocculation was observed in some tubes. Strains in which no O antigen was detected by the standard procedure were grown in broth, autoclaved for 5 h, cooled, and tested with the standard 1:200 dilution of antisera. The two strains that gave positive reactions with antisera to more than one O antigen were retested in twofold serial dilutions (1:400 to 1:3,200) of each antiserum. The O antigen whose antiserum gave the highest titer was considered the strain's O antigen.

Strains in which no H antigen was detected by the standard procedure were tested in two ways. (i) They were retested after multiple transfers through semisolid agar, and (ii) they were tested in various dilutions (1:250, 1:500, 1:750) of antisera, beginning with the dilution more concentrated than the standard procedure. The antiserum giving the highest titer was considered positive. Six strains gave positive reactions to more than one H antigen. Serial dilutions of antisera (1:1,000, 1:2,000, 1:4,000) were tested against these strains, and the antiserum giving the greatest titer was considered positive. To decrease the possibility of error, the titrations to resolve cross-reactions were all done on the same day.

Reproducibility of API. In addition to testing each isolate twice, a single colony of strains tested was plated on 5% sheep blood agar (Scott) for isolation. Five isolated colonies were inoculated into each of five tubes of sterile saline, inoculated in API strips, incubated, and read simultaneously.

RESULTS

Biotyping. Among the 117 *S. marcescens* isolates, 13 biotypes were found. Isolates from the same source in any patients were included in Table 1 only if they were different biotypes. The predominant biotypes were 5 307 721, 5 317 521, and 5 317 721 (Table 1). Only two biochemical tests, urea and inositol fermentation, varied among these three biotypes. The glucose-negative strain (5 303 721) fermented glucose in conventional media and probably did not change the indicator in the glucose cupule of the API strip due to indicator reversion from nitrate reduction (K. Tomfohrde, API, personal communication). Biotype 5 105 121 was both citrate and sorbitol negative and was confirmed as *S. marcescens* by the Connecticut State Health Department and the API laboratories. The isolate fermented sorbitol on conventional media after 7 days of incubation.

Serotyping. Serotypes were determined for 93 isolates, including at least one isolate of each biotype. Isolates from the same patient were included in Table 2 only if they were of different serotypes. The H antigen was undetermined in 11 (13%) strains and the O antigen was undetermined in 6 strains (7%). Among the strains we were able to completely serotype, there were 21 different types. The three most frequent serotypes were O14:H12 (16%), O14:H5 (10%), and O14:H1 (8%).

Comparison of biotyping and serotyping. Of the 117 strains biotyped, 93 strains isolated from 86 patients were serotyped. Comparison of serotypes and biotypes (Tables 1 and 2) indicated that strains that were apparently the same by one method could be subdivided further by the other. For example, 5 315 721, the most prevalent biotype, could be subdivided into nine serotypes, whereas the isolates of the most frequent serotype, O14:H12, were of five biotypes. If each group of strains of the same biotype, including those with an undetermined O or H antigen, is further subdivided on the

TABLE 1. *Distribution of serotypes among S. marcescens biotypes*

Biotype	No. of isolates	%	No. serotyped	No. of different serotypes
5 307 521	6	5	6	4
5 307 561	12	10	8	8
5 307 721	20	17	13	6
5 307 761	12	10	10	5
5 317 521	28	24	20	9
5 317 561	10	9	5	4
5 317 721	18	15	13	10
5 317 761	6	5	6	5
5 105 121	1	1	1	1
5 305 721	1	1	1	1
5 307 720	1	1	1	1
5 303 721	1	1	1	1
5 107 721	1	1	1	1

TABLE 2. *Distribution of biotypes among S. marcescens serotypes*

Serotype	No. of isolates serotyped	No. of different biotypes
O2:H1	6	4
O2:H5	3	2
O4:H1	4	3
O5:H1	3	3
O5:H6	1	1
O6:H1	1	1
O9:H1	1	1
O9:H11	1	1
O10:H1	1	1
O10:H2	1	1
O10:H11	3	3
O10:H12	1	1
O10:H13	1	1
O11:H1	3	2
O13:H4	1	1
O14:H1	7	6
O14:H2	5	2
O14:H4	1	1
O14:H5	9	3
O14:H10	2	2
O14:H12	14	5
O14:H −[a]	9	5
O−:H1	4	4
O5:H −	1	1
O14:H −	1	1
O −:H5	1	1
O −:H2	1	1

[a] −, No reaction with any of the antisera.

basis of serotype, 56 different types of *S. marcescens* can be recognized among the 93 isolates, with three sero-biotypes predominating: 5 317 521, O14:H5 (6%); 5 307 721, O14:H12 (4%); 5 307 761, O14:H12 (4%). Bacteriocin typing (R. Lyons, manuscript in preparation) confirms that these three sero-biotypes are not the same strain and contain a number of bacteriocin types.

Reproducibility of biotyping. Twenty of the patients in this study had *S. marcescens* isolated more than one time from the same or a different site. Twelve were from the same site and eight were from different sites. Isolates of more than one biotype were isolated in seven cases. In four cases (1, 3, 4, and 6) the isolates were different serotypes as well as different biotypes, and in three of these *S. marcescens* was isolated from different sites (Table 3). It appeared that infection with different biotypes occurred in these patients.

Serotypes were not available for the duplicate isolates from patients 2 and 7. The three isolates from patient 2, each a different biotype, were isolated about 2 weeks apart. The two isolates from patient 7 were isolated during separate hospital admissions. The two isolates from patient 5 were the same serotype and bacteriocin type (Lyons, manuscript in preparation), and their biotypes differed by one reaction, the ability to oxidize melibiose. Duplicate biotyping of these isolates was consistent, and the color change in the melibiose cupule was clearly positive for one isolate and clearly negative for the other.

Duplicate testing of all strains gave the same 7-digit number, but in a number of instances the urea and inositol reactions were difficult to score as positive or negative without further incubation. At 18 h 10 of 234 (4%) readings of urease production and 23 of 234 (10%) readings of inositol fermentation were not clearly positive. After an additional 6 to 10 h of incubation, all of these reactions were positive. To resolve the question of reproducibility, two strains with common biotypes and one with a rare biotype were tested to see if their biotypes remained constant. The following biotypes were tested: 5 307 721, 5 317 521, and 5 105 121. In all cases the biotypes were consistently reproducible; however, in some cases the inositol was difficult to interpret. The strains with biotypes 5 317 521 and 5 105 121 were definitely inositol negative, with no color change, but the strains with biotype 5 307 721 were questionable. On close inspection, some of the inositol cupules were yellow-green rather than yellow; on further incubation, they did turn yellow. Since we do not routinely incubate the 20E API strips for more than 24 h, this could cause variation in biotyping.

DISCUSSION

A rapid and simple typing system is needed to study nosocomial outbreaks of *S. marcescens* infection. Biotyping holds the promise of being such a system. It is far less tedious than other

TABLE 3. *S. marcescens with different biotypes isolated from the same patient*

Patient	Source	Biotype	Serotype	
			O	H
1	Toe	5 317 721	10	1
	Stump	5 307 761	10	2
2	Urine	5 317 761	4	1
	Urine	5 317 721	ND[a]	
	Urine	5 317 561	ND	
3	Kidney	5 317 521	14	5
	Urine	5 307 721	14	2
4	Respirator	5 307 721	2	1
	Sputum	5 317 721		1
5	Urine	5 307 761	14	1
	Urine	5 307 721	14	1
6	Urine	5 317 521	14	5
	Urine	5 317 561	14	
7	Urine	5 317 721	15	5
	Urine	5 317 521	ND	

[a] ND, Not done.

methods of typing and involves little added expense for laboratories already using the API system.

Of the 50 biotypes identified as *S. marcescens* and listed in the API analytical index, we found 13 to exist among our 117 strains. Five of these 13 biotypes represented 77% of the strains. We have since found five additional biotypes, but biotypes 5 307 721 and 5 317 521 continue to be the most common.

To further differentiate the 117 strains, serotyping was done with 93 isolates and these were compared with the biotypes. There were 27 different serotypes, and there were several serotypes within each biotype; similarly, most serotypes contained more than one biotype. The 93 strains were divided into 56 combined sero-biotypes. This is similar to the findings of Rennie and Duncan (12), who reported 29 biotypes and 40 capsular types among 270 strains of *Klebsiella*. By combining biotypes and capsular types, they could subdivide their strains into 100 serobiotypes.

There were three predominate sero-biotypes found in our hospital: 5 317 521-O14:H5, and 5 307 761-O14:H12, which were isolated throughout the study, and 5 307 721-O14:H12; these were all found during 1 month and may have been involved in a small hospital outbreak.

There was only minimal variation in biotype on duplicate testing. The principal variation involved the inositol reaction, which was difficult to score as positive or negative without further incubation for 10% of the readings. Butler et al. (3) reported that only 55.5% of 110 *Enterobacteriaceae* gave identical biotypes on repeat testing. Although the information on variation of reactions with each species was not given, they found that urease production and isositol fermentation were, respectively, 95 to 96% and 98 to 99% reproducible for the *Enterobacteriaceae* tested. Rennie and Duncan (12), using conventional biochemical tests, found variations in the biotypes of repeat isolates of *Klebsiella* obtained on more than one occasion from the same patient. Of 37 patients with repeat isolates, 13 (38%) had different biotypes in the second specimen. Eight of these were also a different capsular type. We observed similar variation in the biotypes of *S. marcescens* by using API. The variations in the biotypes and serotypes of isolates from the same source in the same patient may be due to multiple biotypes in an infection or a secondary infection with different strains of the same species.

As a first step in looking at a nosocomial outbreak of *S. marcescens* infection, biotyping can be a useful tool. If two organisms have different biotypes (with the possible exception of the inositol reaction noted above), they are probably different strains, but if they are the same biotype this is not necessarily true. Serotyping and biotyping combined gives a more exact way of determining whether two isolates are the same strain. The addition of a third typing method such as bacteriophage or bacteriocin typing strengthens this classification even more but may be too tedious for the routine laboratory.

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