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Multiple Biotypes of *Klebsiella pneumoniae* in Single Clinical Specimens

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The occurrence of multiple biotypes of *Klebsiella pneumoniae* within single specimens was determined in 59 clinical specimens. Biotyping was performed on five colonies of *K. pneumoniae* from each specimen, using the API 20E system (Analytab, Inc., New York) for identification of *Enterobacteriaceae* with strict adherence to the manufacturer's instructions. Multiple biotypes of *K. pneumoniae* were present in 31% (18) of the clinical specimens. Twenty-eight colonies representative of specimens with single and multiple biotypes were tested further for biotype reproducibility. Whereas genus and species identification was 100% reproducible, variation of one or more biochemical tests on serial transfers resulted in biotype reproducibility of only 64%. The greatest variation in biochemical tests occurred with urease (14%), indole production (10%) and citrate utilization (9%). Multiple biotypes in single specimens appear to be due to both inherent differences among the colonies in the specimen and variability in the system used to determine biochemical reactions. The presence of multiple biotypes limits the usefulness of biochemical typing for epidemiological surveillance of *K. pneumoniae*.

The Analytab Products, Inc. (New York, N.Y.) 20E (API 20E) system for identification of *Enterobacteriaceae* is well evaluated (6, 8), is used routinely in many clinical laboratories, and provides a simple, standardized method for biotyping. There are reports of the usefulness of this system in detecting nosocomial outbreaks of *Erwinia* (F. R. Neblett, E. J. Bottune, and J. F. Eisses, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, C116, p. 46) and *Salmonella* (N. Trowers, L. Camino, B. Beatty, E. Dorvill, F. Jackson, E. Torres, and M. Brimmage, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, C117, p. 46).

During routine use of the API 20E system, we observed different biotypes of the same species in single clinical specimens. This was most apparent with *Escherichia coli* and *Klebsiella pneumoniae*. The present study was undertaken to determine whether multiple biotypes of the same species in single clinical specimens in fact occurred, or whether our observations were due to either errors in the use and interpretation of the API system or lack of reproducibility of the biochemical tests in the system.

MATERIALS AND METHODS

***K. pneumoniae* isolates.** Fifty-nine consecutive specimens, containing pure cultures of *K. pneumoniae* received in the clinical microbiology laboratory of Saint Francis Hospital, Hartford, Conn., over a period of 6 weeks, were studied. The clinical sources of these specimens are listed in Table 1. The majority of the specimens were urine and sputum.

Biotyping. The API micromethod was used for biotyping and included the tests listed in Table 2. The biotype of each of five well-isolated colonies from each plate was determined, strictly adhering to the manufacturer's instructions as reported previously (5). At the same time, each colony was inoculated onto a brain heart infusion agar (BBL) slant and stored at 4°C after 24 h of incubation at 37°C.

Inoculum size and incubation time were carefully controlled. Inoculum size, determined by colony counts on the API saline inoculum, was 0.8×10^7 to 2.0×10^7 colony-forming units/ml. All API strips were incubated for 24 h and read by one of us (M. I. de S.). The biochemical results were converted to a seven-digit number according to the instructions given in the API analytical profile index (1). This profile number is referred to as the biotype.

Reproducibility of API 20E. Twenty-eight colonies, of which five were from specimens with a single biotype and 23 were from 16 specimens with multiple biotypes, were selected to test reproducibility of the API 20E biochemical tests for *K. pneumoniae*. Each colony was transferred from the stored brain heart infusion agar slant to a blood agar plate (Scott Laboratories, Inc., Fiskeville, R. I.) and streaked for isolation. After 24 h of incubation at 37°C, the biotype of a single colony was determined, and at the same time the colony was also restreaked to another blood agar plate. These serial transfers were repeated a total of four times. After each transfer, the API biotype was determined from a single colony.

TABLE 1. *Source of specimens with multiple biotypes*

Source	No. studied	No. with multiple biotypes
Urine	41	10
Sputum	11	7
Wounds	3	1
Blood	2	0
Miscellaneous	2	0

TABLE 2. *API 20E biochemical tests*

Biochemical test	Abbreviation
o-Nitrophenyl-β-D-galactosidase	ONPG
Arginine dihydrolase	ADH
Lysine decarboxylase	LDC
Ornithine decarboxylase	ODC
Citrate utilization	CIT
H_2S production	H_2S
Urease	URE
Tryptophan deaminase	TDA
Indole production	IND
Acetoin production	VP
Gelatinase	GEL
Glucose fermentation	GLU
Mannitol fermentation	MAN
Inositol fermentation	INO
Sorbitol fermentation	SOR
Rhamnose fermentation	RHA
Sucrose fermentation	SAC
Melibiose fermentation	MEL
Amygdaline fermentation	AMY
Arabinose fermentation	ARA

RESULTS

Detection of multiple biotypes. Of the 59 specimens examined, 18 (31%) contained colonies of more than one biotype (Table 1). Although the majority of these were urine isolates, only 24% of the urines tested had multiple biotypes, whereas multiple biotypes occurred in 64% of the sputa tested (Table 1).

The distribution of the number of different biotypes within each of the 18 specimens with multiple biotypes was as follows. There were 11 specimens with 4 colonies of one biotype and 1 colony of a different biotype, 5 specimens with 3 colonies of one biotype and 2 colonies of another, and 2 specimens with 3 different biotypes with 3 colonies of one biotype and 1 each of 2 other biotypes. There were 15 different biotypes found among the 295 individual colonies tested, which are listed in Table 3. More than half (51%) were of the biotype 5 215 773. Biotypes 5 205 773 and 5 255 773 were also common.

Reproducibility of biotypes. At the end of four serial transfers of the 28 colonies, genus and species identification was 100% reproducible. The original biotype was maintained during four transfers by 18 (64%) of the colonies. The remaining 10 colonies changed their biotype because of a variation of either one biochemical reactions (six colonies) or more than one (four colonies). The changes in the biochemical reactions were inconsistent in that in some cases the original colony gave a positive reaction and after one of the transfers the reaction was negative, whereas in others the same biochemical test went from negative to positive. In several instances, the biotype change reverted back to the original biotype after subsequent transfer. The reproducibility of each of the biochemical tests among the total 112 strips tested during four serial transfers of the 28 colonies are listed in Table 4. A total of 47 (2%) biochemical tests among the 2,240 individual tests performed were not reproducible. The greatest variation occurred in the production of urease which had the lowest reproducibility of 86%. Citrate utilization was 91% reproducible, indole production 90%, ornithine decarboxylase 97%, and acetoin production 98%. Others were 100% reproducible. The results at the end of four serial transfers of 16 of the above colonies

TABLE 3. *Frequency of biotypes in 295 colonies*

Biotypes	No. of colonies	%
5 215 773	152	51
5 205 773	51	17
5 255 773	29	10
1 215 773	19	6
5 245 773	11	4
5 215 763	9	3
5 355 773	5	2
5 214 773	4	1
5 015 573	3	
5 217 773	3	
1 205 773	3	
5 005 773	2	<1
5 004 773	2	
5 014 773	1	
5 205 763	1	

TABLE 4. *Reproducibility of API 20E biochemical tests among 112 colonies*

Biochemical test[a]	Reproducibility (%)
ONPG ADH LDC	100
H_2S TDA GEL	100
GLU MAN INO SOR	100
RHA SAC MEL AMY ARA	100
VP	98
ODC	92
CIT	91
IND	90
URE	86

[a] See Table 2 for abbreviations.

that were from 7 specimens that had multiple biotypes are listed separately in Table 5. These 16 colonies were chosen because they represented one colony of each different biotype within each of the 7 specimens. Five of the 7 specimens continued to contain at least two biotypes per specimen at the end of the transfers indicating that more than one stable biotype may occur in a single specimen.

DISCUSSION

An evaluation of 59 clinical isolates of *K. pneumoniae* for the presence of multiple biotypes within the same specimen using the API 20E system demonstrated that 31% of the specimens did contain colonies of more than one biotype. Some of this variation in API profiles of colonies in the same clinical specimen appears to be due to the presence of inherently different cells since five of seven specimens containing multiple biotypes originally, maintained multiple biotypes after serial transfer. On the other hand, some of the variation seen among colonies from single specimens is due to lack of reproducibility within the system.

Recently Butler et al. (2) reported on the reproducibility of the API 20E system with 110 clinical isolates of *Enterobacteriaceae*. They found a high reproducibility of genus-species identification, but overall reproducibility of biotypes was only 55.5%. This is similar to our reproducibility of 64%. They concluded that inoculum size, incubation time, technologist interpretation, and strip variability all affect the API results and must be standardized. In one experiment in which the variable was the number of technologists reading the strips, they found that in 48.7% of replicate readings by four technologists of two API strips prepared from the same bacterial suspension, at least three technologists agreed on one API profile number for one strip and also agreed on a different API profile number for the other strip. They felt this lack of agreement between the two strips was due to strip variability rather than interpretative variability.

TABLE 5. *Reproducibility of 16 colonies with multiple biotypes*[a]

Specimen no.	No. of biotypes in original specimen	Results at end of 4 transfers	
		No. of biotypes	Presence of multiple biotypes
25238	2	1	No
26394	2	2	Yes
26654	2	1	No
33092	2	2	Yes
33739	2	2	Yes
25501	3	2	Yes
23685	3	2	Yes

[a] One colony representative of each biotype was serially transferred four times, and the biotype was determined after each transfer.

We found that even when inoculum size, incubation time, and technologist interpretation were carefully controlled, the urease, indole, citrate, acetoin, and ornithine decarboxylase tests were not 100% reproducible. Butler et al. (2) found that among clinical isolates of *Enterobacteriaceae* only H_2S, indole, mannitol, and tryptophan deaminase were 100% reproducible.

Strip variation does seem to occur, however the particular reactions which vary appears to depend on the species. With *K. pneumoniae*, variation occurs mainly with urease, citrate, and indole, whereas with *Serratia marcescens* variation occurs with urease and inositol. (4) Thus the strip to strip variation one sees may in part be due to the organism being tested.

Some of the variation may not be inherent in the API 20E strips since Rennie and Duncan (4) using a biotyping scheme for *K. pneumoniae* with conventional biochemical tests found biotypes of repeat isolates from 7 out of 24 patients varied due to changes in urease production. The isolates from each patient were the same serotype and were from patients in whom these authors felt cross-infection was not clinically probable. It may be that some biochemical reactions such as urease production vary independently of the detection system.

Five out of 7 (71%) of the specimens we tested contained colonies with different API profile numbers which did not change during four serial transfers, indicating that more than one biotype in a single specimen does occur. Although this has not been previously reported, there are indications that others have made similar observations. Rennie and Duncan (4) observed that repeat isolates from 8 out of 37 patients showed major changes in biotype. They were also of a different capsular type. We (5) obtained similar results when biotypes of *S. marcescens* isolated from 20 patients on more than one occasion were compared. There were seven patients with repeat isolates of different biotypes. Isolates from four of these were also different serotypes.

The presence of colonies in a single clinical specimen which appear to be inherently different biochemically could be due to primary infection with more than one biotype, secondary infection or colonization with a different bio-

type of the same species or changes selected during antibiotic or immunosuppressive therapy. Rennie and Duncan (4) suggest secondary infection is probable since most of their patients with a change in biotype had conditions such as indwelling catheters and tracheostomies. An examination of the length of hospital stay and chemotherapy of the patients from whom specimens were received would help elucidate the origin of multiple biotypes.

It is known that urease and H_2S production may be plasmid-borne. (7) Since spontaneous plasmid loss does occur, perhaps this genetic change may contribute to the occurrence of multiple biotypes.

Thus, the presence of multiple biotypes in single specimens appears to be the result of both inherent differences in the colonies derived from an infecting population and the system used to detect the biochemical reactions. It is difficult to determine what percentage of the colonies in a specimen have inherently different biotypes and what percentage are due to variability of the system used to determine the biochemical reactions.

The increasing frequency with which *K. pneumoniae* is being encountered in nosocomial infections has led to the development of typing methods of this organism that would facilitate epidemiological investigations. The usefulness of capsular serotyping is well documented (3). By using a combination of biochemical and serological typing of clinical isolates of *K. pneumoniae*, Rennie and Duncan (4) were able to demonstrate that the numerical biotypes and serotypes of the strains varied independently and that, when used in conjunction, the two methods subdivided the strains into many more distinct types than either method used alone. They felt numerical biotyping was a useful technique. Since many clinical microbiology laboratories use the API 20E routinely, it could be a useful aid in epidemiological surveys. This would require a high reproducibility rate of the 20 biochemical reactions included in the API strip. Although attempts were made to minimize the technical variables, variation of one or more biochemical reactions on serial transfers resulted in biotype reproducibility of only 64%. Even if the biotypes had been reproducible, the presence of more than one biotype in single specimens makes any biochemical typing scheme useless for the epidemiological surveillance of *K. pneumoniae*.

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