JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 1983, p. 1079–1083
0095-1137/83/111079-05$02.00/0

Vol. 18, No. 5

O Serotyping Scheme for *Enterobacter cloacae*

MICHAEL A. GASTON,* CANDID BUCHER,† AND TYRONE L. PITT

Division of Hospital Infection, Central Public Health Laboratory, Colindale, NW9 5HT, London, England

Received 25 April 1983/Accepted 9 August 1983

A serotyping scheme for *Enterobacter cloacae* based on heat-stable somatic antigens is described. A total of 28 antisera were prepared in rabbits, and titers of agglutinins were high (>640). Some cross-reactions were observed, and 11 sera required absorption before routine use. Of 300 clinical isolates from 66 hospitals, 77.6% were typable, 11.4% were not agglutinated by any of the sera, and 11.0% were autoagglutinable in saline. The eight most frequent serotypes were O3 (21.3%), O8 (13.3%), O1 (7.6%), O13 (5.0%), O9 (4.7%), O10 (3.0%), O16 (3.0%), and O25 (3.0%).

Enterobacter cloacae has been isolated from a wide range of infections in hospitalized patients (8, 15), particularly in neonatal and burn units (10, 14). Among the sources of infection that have been implicated in outbreaks are unsterile infusion fluids (6, 9), pressure transducers (7, 20), hemodialysis equipment (1), and contaminated glass urinals (12). Detailed epidemiological investigations of outbreaks have not been possible owing to the lack of suitable typing schemes, but recently, Freitag and Friedrich (5) and Traub et al. (19) have described bacteriocin typing schemes.

There have been a few limited serological investigations of strains of *Enterobacter* spp., but inconsistencies in the classification of proposed type strains make the results difficult to interpret (4, 17, 18). Sakazaki and Namioka (16) reported on the O and H serology of 170 clinical and veterinary isolates; they distinguished 53 O and 56 H antigens in agglutination tests, but to our knowledge, their serotyping scheme has not been applied to the investigation of outbreaks of infection in hospitals.

We report an O serotyping scheme based on an agglutination test and present the results obtained from typing 300 clinical isolates.

MATERIALS AND METHODS

Bacterial isolates. A total of 327 clinical isolates and 1 veterinary isolate of *E. cloacae* from 64 hospitals in the United Kingdom and 2 in Africa were studied. They were identified by the API 20E system (API Laboratory Products Ltd., Basingstoke, England), and atypical test results were confirmed by conventional biochemical tests (3). All strains were cultured at 37°C for 18 h.

Serum production. Strains were chosen for serum production if they were not typable with existing sera. Vaccines were prepared by inoculating three colonies into either brain heart infusion broth (CM 225; Oxoid Ltd., London, England) or Tryptone Soya broth (CM 129; Oxoid Ltd.). After incubation, cultures were boiled for 1.5 h, and the cells were centrifuged at 5,000 × *g* for 20 min and suspended in 0.85% saline at ca. 4 × 10^9 cells per ml. The opacity corresponded to tube no. 5 (Wellcome Opacity tubes; Burroughs Wellcome Co., Beckenham, England). Adult New Zealand white rabbits were given four intravenous injections of the vaccine, via the marginal ear vein, at 3- to 4-day intervals in the following volumes (ml): 0.5, 1.0, 1.5, and 2.0. Three days after the last injection, a test bleed was taken from the marginal ear vein, and if the titer was greater than 320, the rabbit was exsanguinated the next day.

Serum absorption. Three plates (15 cm in diameter) of diagnostic sensitivity testing agar (CM 261; Oxoid Ltd.) were seeded with a log-phase broth culture of the test strain. After incubation, the cells were harvested in saline and boiled for 1.5 h before centrifugation at 5,000 × *g* for 20 min. The deposit was suspended in 9.5 ml of saline, and 0.5 ml of serum was added to give a final dilution of 1 in 20; the mixture was incubated at 37°C for 1 h and overnight at 4°C. The cells were removed by centrifugation at 12,000 × *g* for 30 min, and the supernatant was filtered through a 0.22-μm nitrocellulose membrane filter (Millipore S.A., Molsheim, France). When necessary, sera were absorbed with a mixture of equal volumes of suspensions of two strains.

Serological tests. Antigens for agglutination tests were prepared by inoculating three colonies of the test strain into 2 ml of Tryptone Soya broth, and after incubation, the cultures were boiled for 1.5 h. The cells were centrifuged at 3,000 × *g* for 30 min and suspended in 4 ml of saline. Serum agglutinin was tested by titration in U-well microtiter trays (Cooke-Microtitre System, Dynatech Laboratories Ltd., Billinghurst, England) with 0.025 ml of antigen and 0.025 ml of serially diluted serum. The trays were incubated at 37°C for 4 h and then at 4°C overnight. The titer of a serum was taken as the highest dilution to give complete agglutination of the bacterial suspension by com-

† Present address: Institut fur Hygiene und Microbiologie, Zurich, Switzerland.

TABLE 1. Homologous and heterologous agglutinin titers of *E. cloacae* O antisera

Antiserum to strain	Agglutinin titers to strain:													
	O1	O2	O3	O4	O5	O6	O7	O8	O9	O10	O11	O12	O13	O14
O1	5,120													
O2	80	1,280												
O3		80	1,280	80										
O4				10,240										
O5					5,120									
O6						1,280	160							
O7						320	2,560							
O8								640	80	80				
O9								160	1,280	1,280	160			
O10								160	320	2,560	80			
O11								80	160	80	5,120			
O12									320	640		1,280		
O13										80			640	
O14														5,120
O15														
O16														
O17														
O18														
O19														
O20														
O21														
O22														
O23														
O24														
O25														
O26														
O27														
O28														

parison with a negative saline control. For routine tests, each serum was used at approximately one-quarter of its homologous titer and incorporated into one of six serum pools; cross-reacting sera were placed in the same pool. Cultures that were agglutinated in a pool were then tested with each of the appropriate component sera.

RESULTS

Biochemical reactions. Fifteen API 20E profiles were found among the clinical isolates, and 259 (79%) had a 3305573 profile.

Sera. In the course of the development of the O serotype scheme, 40 experimental sera were produced in rabbits. Strains for immunization were grown either in brain heart infusion broth or Tryptone Soya broth. No difference in antigenicity was observed between the vaccines in different broths.

The experimental sera were evaluated by cross-agglutination tests in which the sera were titrated with homologous and heterologous antigens. In general, homologous titers were high (640 to 10,240) and heterologous reactions were few, especially among sera with homologous titers above 2,560. Sera exhibiting cross-reactions within four doubling dilutions of the homologous titer were absorbed with the appropriate heterologous strain. When two or more sera showed strong homology by cross-reactions and cross-absorption, only one was selected for inclusion in the provisional serotype scheme. Two sera of low titers (320) were excluded because they required sequential absorption with three or more strains, and this resulted in too great a loss of specific titer.

Twenty-eight sera were selected to form the serotype scheme, and their homologous and heterologous titers are shown in Table 1. Fifteen sera were specific for their homologous strain, and a further two gave heterologous titers which were at least 32-fold lower than the homologous titer and thus did not require absorption. In contrast, types O8, O9, O10, O11, and O12 appeared to represent an antigen group, as sera to these strains agglutinated at least two other members of the group to significant titers. A single absorption was required to render three of these sera specific, but serum O9 needed to be absorbed with strains O8 and O10 (Table 2).

Serotyping of clinical isolates. Of the 300 cultures of *E. cloacae* tested, 77.6% were assigned to a specific O type, 11.4% were not typable (NT) with O sera, and a further 11.0% were autoagglutinable in saline. The distribution of serotypes is shown in Table 3. The most frequent groups were O3 (21.3%) and O8 (13.3%), and overall, eight types accounted for approxi-

TABLE 1—*Continued*

Agglutinin titers to strain:													
O15	O16	O17	O18	O19	O20	O21	O22	O23	O24	O25	O26	O27	O28
2,560													
	10,240												
		1,280	80	160									
			10,240	320									
			320	2,560									
					2,560								
						5,120							
							1,280						
								20,480					
									5,120				
										10,240			
											1,280		
												10,240	
													10,240

mately three-quarters of all the typable isolates. Six sera did not react with any clinical isolates. Each culture was typed twice at an interval of 3 months; no variation of O type was observed, although some strains became autoagglutinable when stored on agar. Three cultures were each agglutinated by two sera, i.e., O1 and O3, O3 and O4, and O16 and O17; however, testing of these strains with dilutions of the appropriate absorbed and unabsorbed sera did not reveal which of the two sero-reactions was due to O-type antigen.

Clinical details were not available for many cultures, but the available information did not indicate a marked relationship between any serotype and the site of isolation.

Titers of field strains with the O-type sera were in general equal to or one-half of the homologous titer, but some strains of serotypes O3 and O8 reacted to only one-quarter of the homologous titer. All cultures which were not agglutinated by the type sera at the dilutions at which they were used and which were, therefore, considered to be NT were tested with

TABLE 2. Agglutination titers of absorbed *E. cloacae* O antisera

Antiserum to strain	Absorbing antigen	Agglutinin titers to strain:									
		O3	O6	O7	O8	O9	O10	O12	O13	O17	O18	O19
O3	O2, O4	640										
O6	O7		1,280									
O7	O6			1,280								
O8	O9				640							
O9	O8, O10					1,280	80					
O10	O9						640					
O12	O9							640				
O13	O10								1,280			
O17	O19									640		
O18	O19										2,560	
O19	O18											640

TABLE 3. Source of *E. cloacae* serotype strains and the distribution of serogroups in 300 clinical isolates[a]

Serum	NCTC reference no.	Pool	Source of vaccine strain	Distribution of serogroup (%)
O1	11570	1	Blood	7.6
O2	11571	1	Nasogastric tube	0.7
O3	11572	5	Blood	21.3
O4	11573	5	NK[b]	2.3
O5	11574	1	Urine	0.0
O6	11575	1	Wound	0.0
O7	11576	1	NK	1.6
O8	11577	2	Spleen	13.3
O9	11578	2	Urine	4.7
O10	11579	2	Blood	3.0
O11	11580	2	Eye	1.0
O12	11581	2	NK	1.3
O13	11582	3	Wound	5.0
O14	11583	3	Blood	0.7
O15	11584	3	Urine	0.7
O16	11585	3	Cecum[c]	3.0
O17	11586	4	NK	0.0
O18	11587	4	NK	0.0
O19	11588	4	NK	1.0
O20	11589	4	Sputum	1.6
O21	11590	4	NK	0.7
O22	11591	5	NK	0.7
O23	11592	5	Nasogastric feed	0.7
O24	11593	6	Throat	2.3
O25	11594	6	Irrigation fluid	3.0
O26	11595	6	NK	0.0
O27	11596	6	Sputum	0.7
O28	11597	6	NK	0.0

[a] Multireactive isolates, 1.0%; NT isolates, 11.4%; autoagglutinable isolates, 11.0%.
[b] NK, Not known.
[c] Veterinary strain.

lower dilutions of all sera to confirm the absence of a type reaction. Two-thirds of the NT cultures were agglutinated by at least one unabsorbed serum to a titer of 160, but no distinct type reactions were observed. Furthermore, one-half of the low titer reactions obtained with NT cultures occurred in sera to the O8 to O12 group.

DISCUSSION

Two recent surveys of infection in hospitals showed that *Enterobacter* spp. accounted for about 4 and 1.1% of infections in the United States and the United Kingdom, respectively (2, 11). In our experience, *E. cloacae* is markedly more frequently associated with infections than are other members of the genus. To facilitate epidemiological studies, we have developed a basic serotyping scheme comprising 28 heat-stable antigen types.

Sera were relatively easy to prepare in rabbits, high homologous titers were obtained, and heterologous agglutinins were readily removed by absorption.

In the course of the study, we observed that strains sometimes became autoagglutinable after prolonged storage on agar media in the laboratory, and so we maintained our type strains in liquid nitrogen. Thus, the frequency of autoagglutinable isolates (11.0%) may be exaggerated as a result of storage.

Autoagglutination in members of the family *Enterobacteriaceae* is generally associated with the expression of an incomplete (rough) lipopolysaccharide antigen. In *Escherichia coli* and *Salmonella* spp. antigenically rough strains generally give a rough colonial morphology. We have not observed an association between morphology and autoagglutination in *E. cloacae*.

The preparation of additional O sera was indicated, since 11.4% of all isolates were NT with the basic serotype set. We raised sera to two of the NT strains but found the levels of cross-reacting antibody and the low homologous titers to be unacceptable for use. This may indicate that a proportion of NT strains do not express satisfactory heat-stable somatic antigens. We observed no association between overt production of capsular polysaccharide on agar media and typability, and at present, we have no evidence to suggest that masking of O antigen by capsules influences O typability.

As high-titer-specific sera to members of the cross-reacting group O8 to O12 could be prepared by absorption, we have decided to retain distinct type numbers for these sera. Nevertheless we appreciate that these types may prove to be minor factors of a major antigen. Therefore, further studies are necessary to determine the immunochemical basis of these and the other type antigens.

We are not able to discuss the hospital epidemiology of *E. cloacae* as most of the isolates typed in this study came from laboratory collections, and relatively little epidemiological information was available to interpret the results. However, we are applying the scheme to recent clinical isolates and would be pleased to receive isolates from possible incidences of cross-infection. Other typing methods are being developed in this laboratory to improve type discrimination, particularly O3 and O8.

Parker et al. (13) suggested that the discriminatory power of a typing scheme is inversely related to the frequency of the largest group. The serotype scheme presented here appears to be adequately discriminatory in that it divided the majority of the strains into clearly defined types. Typing of all strains on two occasions indicated that the system was reproducible in the laboratory. Nevertheless, an accurate measure of both discrimination and reproducibility

will only be obtained when the O scheme is compared with other systems such as bacteriocin (4, 19) or bacteriophage typing.

ACKNOWLEDGMENTS

We thank the Computer Trials Laboratory, Colindale, London, S. W. B. Newsom and A. King for providing many clinical isolates.

The type strains described in this paper are available from The National Collection of Type Cultures, Colindale, London.

LITERATURE CITED

1. **Borobio, M. V., and E. J. Perea.** 1975. Bacterihemias por *Enterobacter cloacae* en enfermos dializados. Rev. Clin. Esp. **137**:517–520.
2. **Centers for Disease Control.** 1981. National nosocomial infections study report, p. 1–14. Centers for Disease Control, Atlanta.
3. **Cowan, S. T.** 1974. Manual for the identification of medical bacteria, p. 137–160. Cambridge University Press, Cambridge.
4. **Deacon, W. E.** 1952. Antigenic study of certain slow lactose fermenting *Aerobacter cloacae*. Proc. Soc. Exp. Biol. Med. **81**:165–170.
5. **Freitag, V., and O. Friedrich.** 1981. Bacteriocin typing of *Enterobacter cloacae* strains. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A **249**:63–75.
6. **Handy, G. H., and J. H. Ackerman.** 1973. Septicemias associated with contaminated intravenous fluids. Morbid. Mortal. Weekly Rep. **22**:99.
7. **Higgins, M. V., and N. King.** 1975. Transducer-associated bacteremia. Morbid. Mortal. Weekly Rep. **24**:295.
8. **John, J. F., R. J. Sharbaugh, and E. R. Bannister.** 1982. *Enterobacter cloacae*: bacteremia, epidemiology and antibiotic resistance. Rev. Infect. Dis. **4**:13–28.
9. **Maki, D. G., F. S. Rhame, D. C. Mackel, and J. V. Bennet.** 1976. Nationwide epidemic of septicemia caused by contaminated intravenous products. Am. J. Med. **60**:471–485.
10. **Mayhall, C. G., V. A. Lamb, W. E. Gayle, and B. W. Haynes.** 1979. *Enterobacter cloacae* septicemia in a burn center: epidemiology and control of an outbreak. J. Infect. Dis. **139**:166–171.
11. **Meers, P. D., G. A. J. Ayliffe, A. M. Emmerson, D. A. Leigh, R. T. Mayon-White, C. A. Mackintosh, and J. L. Stronge.** 1981. Report on the national survey of infection in hospitals. J. Hosp. Infect. **2**(Suppl.):13–17.
12. **Mummery, R. V., B. Rowe, and R. J. Gross.** 1974. Urinary-tract infections due to atypical *Enterobacter cloacae*. Lancet **ii**:1333.
13. **Parker, M. T., T. L. Pitt, E. Asheshov, and D. R. Martin.** 1976. The selection of typing methods for *Pseudomonas aeruginosa*, p. 469–486. *In* Proceedings of the VI international colloquium on phage typing and other laboratory methods for epidemiological surveillance, vol. 2. Gesellschaft für Mikrobiologie und Epidemiologie der DDR, Wernigerode, Germany.
14. **Powell, J., M. A. Bureau, C. Pare, M. L. Gaildry, D. Cabana, and H. Patriquin.** 1980. Necrotizing enterocolitis. Am. J. Dis. Child. **134**:1152–1154.
15. **Rose, H. D., and M. L. Koch.** 1966. Hospital-acquired *Aerobacter cloacae* infections. Arch. Intern. Med. **117**:92–98.
16. **Sakazaki, R., and S. Namioka.** 1960. Serological studies on the *Cloacae* (*Aerobacter*) group of enteric bacteria. Jpn. J. Med. Sci. Biol. **13**:1–12.
17. **Sedlak, J., and D. Matejovska.** 1958. On the taxonomy and systematics of the *Aerobacter cloacae* groups (*Cloacae cloacae*). J. Hyg. Epidemiol. Microbiol. Immunol. **2**:285–299.
18. **Stuart, C. A., K. M. Wheeler, R. Rustigian, and A. Zimmerman.** 1943. Biochemical and antigenic relationships of the paracolon bacteria. J. Bacteriol. **45**:101–119.
19. **Traub, W. H., M. Spohr, and R. Blech.** 1982. Bacteriocin typing of clinical isolates of *Enterobacter cloacae*. J. Clin. Microbiol. **16**:885–889.
20. **Weinstein, R. A., W. E. Stamm, L. Kramer, and L. Corey.** 1976. Pressure monitoring devices. J. Am. Med. Assoc. **236**:936–938.