JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 1995, p. 8–10 Vol. 33, No. 1
0095-1137/95/$04.00+0

Use of Yeast Killer System To Identify Species of the *Nocardia asteroides* Complex

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Received 28 June 1994/Returned for modification 1 August 1994/Accepted 30 September 1994

We evaluated the ability of the yeast killer system to differentiate members belonging to the *Nocardia asteroides* complex (*Nocardia asteroides*, *Nocardia farcinica*, and *Nocardia nova*). *Nocardia* strains were selected randomly from clinical isolates. Type strains of each *Nocardia* species and recognized killer yeasts were taken from different collections. A clear area of inhibition surrounding the yeast cells demonstrated a positive killer effect on *Nocardia* spp. Two yeast strains, *Pichia mrakii* (K9) and *Pichia lynferdii* (K76), showed different killer activities against each *Nocardia* species. The group *N. asteroides* was identified as $K9^+$ $K76^-$, the group *N. farcinica* was identified as $K9^-$ $K76^-$, and the group *N. nova* was identified as $K9^+$ $K76^+$. The three killer identifications correlated with specific taxonomic groups determined by using classical methods. The yeast killer system may be a useful means for identifying organisms within the *N. asteroides* complex.

Many studies have shown the heterogeneity of the strains identified as *Nocardia asteroides* (5, 14). By using different approaches, including antigen-induced delayed hypersensitivity (9), numerical taxonomy and DNA-DNA reassociations (6, 8, 19), and antimicrobial susceptibility (17), strains of *N. asteroides* were separated into *N. asteroides* sensu stricto and *Nocardia farcinica*. Additional numerical studies established a further subdivision of *N. asteroides* sensu stricto into *N. asteroides* new sense and *Nocardia nova* (20). Current methods of identification of *Nocardia* species, such as pattern of decomposition of particular substrates or acid production from all carbohydrates except rhamnose, are unable to distinguish between these three species (i.e., *N. asteroides*, *N. farcinica*, and *N. nova*). Because of their similarity, they were considered members of the *N. asteroides* complex (21).

However, their epidemiological (2), pathogenic (4), and antibiotic susceptibility patterns seem different (21, 22) and could lead to distinct management of infected patients and clinical outcome (2). It is, therefore, important to evaluate an easy and quick method to differentiate between species of the *N. asteroides* complex.

It was proved that the killer phenomenon, which is a secretion process of extracellular (glyco)proteins (killer toxins) by self-immune killer yeasts, was widespread among microorganisms (15). After a promising study that provided differentiation of strains belonging to the *N. asteroides* complex, *Nocardia brasiliensis*, and *Nocardia otitidiscaviarum* (13), we decided to evaluate this system in its ability to separate species of the *N. asteroides* complex.

MATERIALS AND METHODS

Organisms. The *Nocardia* strains used in this study were selected randomly from clinical isolates received for identification in the National Reference Center for Mycosis and Antifungal Agents, Institut Pasteur, Paris, France, and belonging to the *N. asteroides* complex. Other strains were kindly provided by the National Chubu Hospital (Obu, Aichi, Japan) from the collection of M. Tsukamura's. As a reference, type strains of each species from the American Type Culture Collection (Rockville, Md.) were used. All *Nocardia* strains were preserved in a sterile saline solution at room temperature.

The killer yeasts were taken from different collections. They were stocked in sterile distilled water at room temperature (Table 1).

Laboratory identification. Actinomycete strains were identified by classical methods (3, 7). Briefly, the genus *Nocardia* was identified by characterization of parietal amino acids (18), cell saccharides (11), and specific mycolic acid (12). Identification of strains to the species level was performed by use of several physiological tests and was based on their growth on particular carbon substrates, such as acetamide, 1,3-butylene glycol, 2,3-butylene glycol, citrate, ethanol, monoethanolamine, and L-rhamnose and their specific enzymatic activities (acetamidase, allantoinase, and arylsulfatase production to 14 days and benzamidase, catalase, pyrazinamidase, and nitrate reduction) (2, 20). The antibiotic resistance phenotype was also included in the identification protocol (2, 21, 22).

Killer activity testing. Sabouraud agar, Sabouraud agar modified (buffered at pH 5.5 ± 0.1), and Sabouraud dextrose broth (Difco Laboratories, Detroit, Mich.) were the media used in this work. The *Nocardia* isolates and the killer yeasts were maintained on Sabouraud agar slant tubes.

At first, one isolated colony of *Nocardia* isolates, grown 1 week at 25°C on Sabouraud agar slant tubes, was inoculated into flasks with 100 ml of Sabouraud dextrose broth plus 1% of Tween 40. The incubation was done for 5 days at 37°C under shaking conditions (70 rpm). After this time, 1 ml of broth culture was mixed with 20 ml of melted Sabouraud agar modified and poured into a petri dish (diameter, 90 mm). After solidification, each killer yeast strain, grown for 48 h at 25°C on Sabouraud agar plates, was streaked for confluency as a line by a common loop application onto the agar surface. The plates were then incubated at 25°C until visible growth of *Nocardia* spp. and yeast cells had been observed.

RESULTS

After approximately 5 days of incubation, the results of the dishes were read. A clear area of inhibition surrounding the streaked yeast cells demonstrated a positive killer effect from the yeast (Fig. 1 and 2) Two killer yeasts (K9 [*P. mrakii* Ahearn WC51] and K76 [*P. lynferdii* NRRL Y-7723]) were selected by their differential activities against the strains belonging to the species of the *N. asteroides* complex investigated among all the ones tested which showed a variable killer activity devoid of capacity of discrimination.

The group *N. asteroides* was identified as $K9^+$ $K76^-$, the group *N. farcinica* was identified as $K9^-$ $K76^-$, and the third group corresponding to *N. nova* was identified as $K9^+$ $K76^+$ (Table 2).

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TABLE 1. Killer yeasts investigated for potential activity against *Nocardia* isolates

Code	Species	Source[a]	Strain no.
K1	*Pichia* sp.	Stumm	1034
K2	*Pichia* sp.	Stumm	1035
K4	*Pichia anomala*	CBS	5739
K9	*Pichia mrakii*	Ahearn	WC51
K36	*Pichia anomala*	UP	25F
K37	*Pichia mrakii*	UCSC	255
K41	*Saccharomyces cerevisiae*	Kandel	SC8
K61	*Pichia americana*	NRRL	Y-2156
K62	*Pichia angusta*	NRRL	Y-2214
K63	*Pichia anomala*	NRRL	Y-366
K67	*Pichia ciferrii*	NRRL	Y-1031
K68	*Pichia dryadoides*	NRRL	Y-10990
K69	*Pichia euphorbiaphila*	NRRL	Y-12742
K76	*Pichia lynferdii*	NRRL	Y-7723
K79	*Pichia muscicola*	NRRL	Y-7005
K80	*Pichia petersonii*	NRRL	YB-3808
K81	*Pichia populi*	NRRL	Y-12728
K82	*Pichia silvicola*	NRRL	Y-1678

[a] Source abbreviations: Stumm, C. Stumm, University of Njimegen, Njimegen, The Netherlands; CBS, Centraalbureau voor Schimmelcultures, Baarn, The Netherlands; Ahearn, D. G. Ahearn, Georgia State University, Atlanta, Ga.; UP, Istituto di Microbiologia, Universitá Cattolica del Sacro Cuore, Rome, Italy; UCSC, Istituto di Microbiologia, Universitá degli Studi di Parma, Parma, Italy; Kandel, J. Kandel, California State University, Fullerton, Calif.; NRRL, Northern Regional Research Laboratory, Peoria, Ill.

DISCUSSION

Although a variety of *Nocardia* spp. can cause human infections (10), strains belonging to the *N. asteroides* complex were the pathogen in the majority of cases reported (1).

Differentiation of the major human *Nocardia* species (*N. asteroides*, *N. brasiliensis*, and *N. otitidiscaviarum*) is currently done on the basis of the pattern of decomposition of various substrates, such as tyrosine, xanthine, adenine, hypoxanthine, or casein (7). However, these tests produce identical results among *N. asteroides* complex species. These are laboriously differentiated by using uncommon methods that are not readily used in current clinical laboratories. *N. farcinica* and *N. asteroides* new sense may be separated by the ability of the former to grow at 45°C, to grow on rhamnose, 2,3-butylene glycol, and 1,2-propylene glycol as a sole carbon source, and to use acetamide as a nitrogen and carbon source (7). *N. nova* can be differentiated from *N. asteroides* by arylsulfatase, α-esterase, and β-esterase activities and the ability to utilize isobutanol as a sole carbon source.

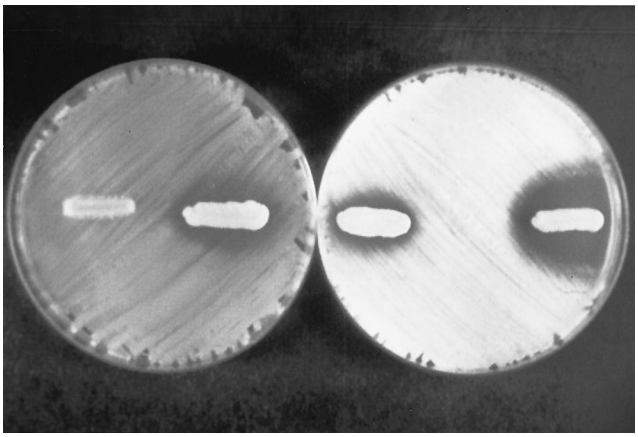

FIG. 1. Identification of *N. asteroides* 90-0318 (left plate) and *N. nova* KK71-19 (right plate) by the killer yeasts *P. lynferdii* K76 (left streak) and *P. mrakii* K9 (right streak).

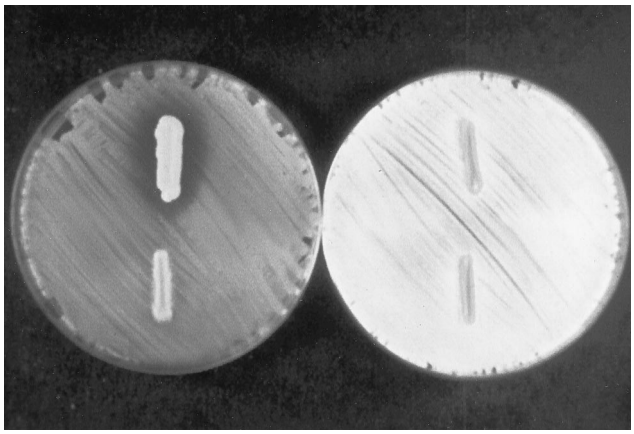

FIG. 2. Identification of *N. asteroides* 90-0598 (left plate) and *N. farcinica* KK73-11 (right plate) by the killer yeasts *P. mrakii* K9 (top streak) and *P. lynferdii* K76 (bottom streak).

TABLE 2. Susceptibility of *Nocardia* species belonging to the *N. asteroides* complex to the killer yeasts

Species and strain no.	Collection[a]	Active killer yeasts	
		K9	K76
N. asteroides new sense			
90-0318	NRCMAA	+	−
90-0598	NRCMAA	+	−
90-0935	NRCMAA	+	−
90-0964	NRCMAA	+	−
KK71-09 (23008)	NCH	+	−
KK71-11 (23046)	NCH	+	−
KK71-14 (23099)	NCH	+	−
19247[b]	ATCC	+	−
N. farcinica			
90-0360	NRCMAA	−	−
90-0769	NRCMAA	−	−
90-0890	NRCMAA	−	−
90-0923	NRCMAA	−	−
90-1104	NRCMAA	−	−
KK73-15 (23013)	NCH	−	−
KK73-14 (23036)	NCH	−	−
KK73-11 (23098)	NCH	−	−
3318[b]	ATCC	−	−
N. nova			
90-0099	NRCMAA	+	+
90-0969	NRCMAA	+	+
90-0983	NRCMAA	+	+
90-1267	NRCMAA	+	+
KK71-08 (23006)	NCH	+	+
KK71-19 (23019)	NCH	+	+
KK71-21 (23096)	NCH	+	+
33726[b]	ATCC	+	+
33727	ATCC	+	+

[a] Collection abbreviations: NRCMAA, National Reference Center for Mycosis and Antifungal Agents; NCH, National Chubu Hospital (M. Tsukamura's collection); ATCC, American Type Culture Collection.
[b] Type strain.

Indeed, reports of microbial analysis in which distinction of species of the *N. asteroides* complex was made showed that none of these species occurred too unfrequently to be neglected. Of 117 *N. asteroides* complex organisms analyzed between 1979 and 1992 by Desmond and Flores (4), 55 (47%) were *N. asteroides* new sense, 34 (29%) were *N. farcinica*, and 28 (24%) were *N. nova*. In the reidentification study of 26 Japanese strains isolated from clinical cases, 11 (42%) were *N. asteroides* new sense, 8 (31%) were *N. farcinica*, and 7 (27%) were *N. nova* (23). Recently, an increasing incidence of infections due to *N. farcinica*, particularly in AIDS patients, was recently reported in Germany (16) and France (2).

The greater virulence of *N. farcinica* within species of the *N. asteroides* complex has been reported (4). By use of a mouse model with intravenous inoculation, the 50% lethal doses for *N. farcinica* were significantly lower than those of the other two species. The analysis of the specimen sites from which the organisms were isolated showed that isolates derived from blood, brain, or bone marrow were more likely to be *N. farcinica* than the two other species.

Wallace et al. (21, 22) separated *N. asteroides* new sense, *N. farcinica*, and *N. nova* by the antimicrobial susceptibility patterns. Cefotaxime-, cefamandole-, and tobramycin-resistant strains matched previous descriptions and reference strains of *N. farcinica*. Isolates characterized by susceptibility to ampicillin and erythromycin were identical to the type strain and previous descriptions of *N. nova*. The susceptibility patterns to these five antibiotics provide a good suggestion as to species identification but must be combined with additional tests, such as growth at 45°C for 3 days, utilization of acetamide as a nitrogen and carbon source, acid production from rhamnose, and arylsulfatase production after 14 days, for diagnostic confirmation. However, the latter tests are not readily available to the clinical laboratory.

The results obtained in this study confirm that killer activity of yeasts operates on other microorganisms and particularly on *Nocardia* spp. This is the first report on the use of the killer system for the identification of species rather than for strain differentiation. In this study, the yeast killer system has permitted the distinction of the three species of the *N. asteroides* complex. The species distinguished were in accordance with the identification of the strains in laboratory collections by use of classical techniques on the basis of their growth capacity on particular carbon substrates and their specific enzymatic activities (3). The yeast killer system could be recommended to laboratories as a means to identify quickly and occurately species of the *N. asteroides* complex. An antibiogram, which must be performed in the clinical laboratory, may provide further confirmation of identification.

In comparison with more conventional methods, the killer system proposed for the identification of species belonging to the *N. asteroides* complex may be of value for the intrinsic reasons of economy, rapidity, and feasibility; these reasons make this system attractive for any laboratory, including those in nondeveloped countries which, like other countries, presumably might be involved in the diagnosis of nocardiosis.

ACKNOWLEDGMENTS

L.P. was supported in his study by grants from the National AIDS Project (Istituto Superiore di Sanità, Ministero della Sanità; no. 7205117) and the F.A.T.M.A. subproject Etiology of Infectious Diseases (no. 93 0062 341/115 15897).

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