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Characterization and serological classification of a collection of *Proteus penneri* clinical strains

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Introduction: Bacteria of the genus *Proteus*, which are a common cause of urinary tract infections, are divided into four species: *P. mirabilis*, *P. vulgaris*, *P. penneri*, and *P. hauseri*, and three unnamed genomospecies, *Proteus* 4, 5, and 6 (single-strain species *P. myxofaciens* was isolated from the gypsy moth). Establishing the serological classification of these species would aid in completing the classification scheme of the whole genus *Proteus* and in applying serological methods in diagnostic procedures and epidemiological investigations for these opportunistic pathogens. The aim of this research was a serological characterization and classification of 57 *Proteus penneri* clinical strains, isolated from patients from different countries all over the world, into *Proteus* O serogroups.

Materials and Methods: Purified lipopolysaccharides (LPSs) extracted from 57 *P. penneri* strains were used as antigens in enzyme immunosorbent assay (EIA), SDS/PAGE, and Western blot techniques, and alkali-treated LPSs in passive immunohemolysis test (PIH), inhibition of PIH, and absorption of rabbit polyclonal O-antisera.

Results: That result confirms the serological distinction of this species within the genus *Proteus*, and may have diagnostic significance.

Conclusions: As a result of serological studies of LPSs extracted from the *P. penneri* strains, one new *Proteus* serogroup, represented by the *P. penneri* 97 strain, was established. Three further strains were classified into the *Proteus* serogroup O8, which had not contained any *P. penneri* strains before. All the remaining strains were classified into 11 already existing *Proteus* O serogroups. It is important to emphasize that 72% of studied strains were classified into serogroups that contain *P. penneri* strains only.

Key words: ***Proteus penneri*** **• O antigen; lipopolysaccharide • O serogroups • O serotyping • serological classification**

Abbreviations: **EIA** – enzyme immunosorbent assay, **LPS** – lipopolysaccharide, **PAGE** – polyacrylamide gel electrophoresis, **PIH** – passive immunohemolysis.

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INTRODUCTION

The medically important bacteria of genus *Proteus*, which are a common cause of urinary tract infections, are divided into four species: *P. mirabilis*, *P. vulgaris*, *P. penneri*, and *P. hauseri*, and three unnamed genomospecies, *Proteus* 4, 5, and 6[13, 14].

Proteus penneri, formerly known as *Proteus vulgaris* biogroup 1, or as indole-negative *P. vulgaris*, was distinguished as a new species within the *Proteus* genus in 1982[6]. These opportunistic pathogens have been isolated mainly from the urine of patients with abnormalities in the urinary tract, and from stool. Identification of the species in routine clinical work only includes phenotypic methods based on biochemical tests. Rapid typing systems are used for neither diagnostic nor epidemiological investigations[13]. One of the main reasons for this is the lack of a complete serological classification scheme for the genus *Proteus*.

The prevailing Kauffmann and Perch scheme of 49 *Proteus* O serogroups includes *P. mirabilis* and *P. vulgaris* strains only[7]. However, it is not complete even in regard to these two species, as Larsson et al.[12] and Penner and Hennessy[16] differentiated 6 and 11 new *Proteus* O serogroups, respectively. Furthermore, a recent investigation by Sidorczyk's group [1, 4, 9, 17, 19, 20, 25–27] has broadened the list of *Proteus* O serogroups by 13 additional new serogroups consisting of *P. penneri* strains only. Such results have proved the serological diversity of this species within the genus *Proteus*. The growing number of *P. penneri* clinical isolates from patients has enhanced its medical significance and suggests the possibility of further new *Proteus* O serogroups consisting of *P. penneri* strains.

Completing the serological classification scheme opens the way to using serological methods in the diagnostics of and epidemiological studies on *Proteus* bacilli. Serotyping is considered to be a simple, cheap, and precise method for the rapid identification of bacteria, and it makes investigations aimed at defining the sources and ways of the spread of infections, especially nosocomial, easier. That is why studies on the re-evaluation of the classification scheme for the pathogenic bacteria *Serratia marcescens* are conducted[2]. The authors have established 19 O serogroups and 14 K serogroups for these microorganisms. Japanese groups have broadened the existing scheme of serological classification of different *Vibrio* species[5, 18]. Pantophlet et al.[15] elaborated the O serotyping scheme for *Acinetobacter* – opportunistic pathogens involved in hospital-acquired infections. The scientists believed that O serotyping is a simple method for the routine identification of these bacteria in clinical laboratories.

The aim of our study was a serological characterization of a collection of 57 *P. penneri* strains isolated from patients in many countries worldwide. The results of these investigations will complete the serological classification of the *P. penneri* species and, in turn, broaden the scheme for the whole *Proteus* genus.

MATERIALS AND METHODS

Bacterial strains

The 57 studied *P. penneri* strains were clinical isolates from patients in different countries (Table 1). Identification of all the investigated strains was confirmed using API 20E tests (bioMérieux, France). The bacterial strains were grown on nutrient broth with 1% glucose. Dry bacterial mass was obtained from an aerated culture by centrifugation of the bacterial cells, washing with distilled water, and lyophilization.

Antigens and serological assays

The lipopolysaccharides (LPSs) were extracted from the dried bacterial cells using a phenol-water procedure[24]. Crude antigens were purified by treatment with aqueous 50% trichloroacetic acid[29]. Alkali-treated LPSs were prepared by saponification with 0.25 M NaOH (56°C, 2 h) and precipitation with ethanol. Purified LPSs were used as antigens in the enzyme immunosorbent assay (EIA), polyacrylamide gel electrophoresis (SDS/PAGE), and Western blot techniques, and alkali-treated LPSs in passive immunohemolysis (PIH) test, inhibition of PIH, and absorption of antisera. All the tests were made according to the procedure described in detail elsewhere[21].

Rabbit antisera

Polyclonal O-antisera were obtained by immunization of rabbits with heat-inactivated bacteria, as described previously[28].

RESULTS AND DISCUSSION

The serological investigations of the LPSs extracted from the 57 studied *P. penneri* strains were performed in two phases. As a preliminary step, the reactions were checked between the LPSs and the group of 22 polyclonal rabbit antisera specific to the *P. penneri* strains representing the *Proteus* O serogroups and

Table 1. Origins and sources of studied 57 *P. penneri* strains

Strain number	Reference number	Source	Origin	Kindly provided by
46	CCUG 3794=990714	blood	Linköping, Sweden	Dr. E. Falsen (Department of Clinical Bacteriology, Göteborg, Sweden)
49	357 Hly$^+$ (hemolysine-positive)	wound	Pécs, Hungary	Dr. L. Emödy (Department of Medical Microbiology and Immunology, Pécs, Hungary)
76	15 BK	urine	Łódź, Poland	our collection
77	E 46	feces	Braunschweig, Germany	Dr. B. Senior (Department of Medical Microbiology, Dundee, UK)
78	E 100	feces	Braunschweig, Germany	
79	E 180	feces	Braunschweig, Germany	
80	E 1054	feces	Braunschweig, Germany	
81	E 1092	feces	Braunschweig, Germany	
82	E 1152	feces	Braunschweig, Germany	
83	E 1264	feces	Braunschweig, Germany	
84	H 727	feces	Braunschweig, Germany	
85	H 956	feces	Braunschweig, Germany	
86	H 1209	feces	Braunschweig, Germany	
87	H 1263	feces	Braunschweig, Germany	
88	H 1286	feces	Braunschweig, Germany	
89	H 1298	feces	Braunschweig, Germany	
90	17518 R	urine	Dundee, UK	
91	T77	thorax wound	Istanbul, Turkey	
92	05665 V	urine	Dundee, UK	
93	16803 Z	urine	Dundee, UK	
94	H 1360	feces	Braunschweig, Germany	
95	E 587	feces	Braunschweig, Germany	
96	E 1263	feces	Braunschweig, Germany	
97	E 1125	feces	Braunschweig, Germany	
100	NTCT 11972=CL 50/81	blood	Newcastle, UK	Dr. B. Holmes (National Collection of Type Cultures, London, UK)
101	CL 189/90=122 B	urine	Bristol, UK	
102	CL 190/90=41 B	pus	Bristol, UK	
104	CL 192/90=30 N	ear swab	Newcastle, UK	
105	CL 193/90=28 N	feces	Newcastle, UK	
106	CL 225/90=CDC 1230-77	urine	Virginia, USA	
107	CL 226/90=CDC 1229-77	sacral ulcer	Virginia, USA	
108	CL 227/90=CDC 2612-80	infected foot	Maryland, USA	
109	CL 232/90=CDC 4020-85	blood	Netherlands	
110	CL 234/90=CDC 1173-71	nasal suction machine	New York, USA	
111	CL 235/90=CDC 1419-81	stool	France	
112	CL 236/90=CDC 4045-85	urine	Virginia, USA	
113	CL 237/90=CDC 4026-86	urine	Puerto Rico	
114	CL 238/90=CDC 1197-81	urine	Florida, USA	
115	CL 242/90=CDC 4455-72	urine	North Carolina, USA	
116	CL 244/90=CDC 4331-83	urine	New Mexico, USA	
117	ISL 695/91=77 B	urine	Bristol, UK	
118	ISL 697/91=29 N	vaginal swab	Newcastle, UK	
119	ISL 698/91=P 377	ulcer	Newcastle, UK	
120	ISL 699/91=P 378	urine	Bristol, UK	
121	ISL 700/91=P 379	urine	Bristol, UK	
122	ISL 701/91=SJP/A/056	urine	Newcastle, UK	
123	ISL 702/91=SJP/A/090	urine	Newcastle, UK	
125	ISL 707/91=CDC 4455-72	urine	North Carolina, USA	
126	ISL 742/91	leg ulcer	Riyadh, Saudi Arabia	
127	1781	unknown	Ontario, Canada	Dr. M. Valvano (Department of Microbiology and Immunology, Ontario, Canada)
128	2175	unknown	Ontario, Canada	
129	3087	unknown	Ontario, Canada	
130	6602	unknown	Ontario, Canada	
131	8838	unknown	Ontario, Canada	
133	35	unknown	Palermo, Italy	Dr. G. Giammanco (Institute of Hygiene and Prevention Medicine, Catania, Italy)
134	60	unknown	Paris, France	
136	63	unknown	Nantes, France	

subgroups which contain *P. penneri* bacilli. In this way, over 1 200 serological systems were analyzed with the PIH test. In further work, only strong cross--reactions (titers 1:12'800–1:102'400), which were comparable to those in homologous systems, were taken into account. Weaker reactions (titers <1:12'800) were omitted, as they only suggested similarity, not identity, of the reacting antigens.

Almost every investigated LPS (53 out of the 57) demonstrated strong cross-reactions with one of the 22 antisera used. However, the four remaining LPSs, namely those of *P. penneri* 97, 106, 107, and 112, did not demonstrate strong reactions with any of the 22 antisera, indicating that these strains did not belong to any O serogroup containing *P. penneri* bacteria. Antisera for two of them, *P. penneri* 97 and 107, were obtained and tested with all four of the non-cross--reacting LPSs and, additionally, with the LPSs of the *P. mirabilis* and *P. vulgaris* strains representing 49 *Proteus* O serogroups of the Kauffmann and Perch scheme (data not shown). From these investigations it emerged that *P. penneri* 97 is serologically distinct from all the *Proteus* strains, because *P. penneri* 97 O-antiserum did not react on a significant level with any of the *Proteus* LPSs (an exception was the homologous one). Therefore, this strain is a candidate for a new *Proteus* serogroup, in which it will appear as the single representative.

P. penneri 107 antiserum reacted strongly with the LPSs of *P. penneri* 106 and 112, *P. vulgaris* O8 (17/57), and the homologous one.

The LPSs studied formed 12 groups of cross-reacting antigens. These 12 systems were selected for further serological studies, performed in the second phase of work, using different, precise serological methods: EIA, inhibition of EIA and PIH in the homologous systems, repeated PIH after absorption of the antisera with the LPSs, and SDS/PAGE – Western blot analyses. The results of the first and the second parts of the study are compiled in Table 2. The results obtained by Western blot are shown in Fig. 1.

High reciprocal titers in PIH and EIA of the respective antisera and strong inhibitory activity of cross--reacting antigens (columns 5–6) prove that the analyzed LPSs belong to 12 different *Proteus* O--serogroups (column 2). This conclusion is confirmed by the results of PIH in the homologous systems after the absorption of the antisera by cross-reacting LPSs, which completely (or almost completely) removed reacting antibodies from the antisera (column 7). All the O-antisera also reacted strongly in Western blot analyses, comparably to the homologous systems, with slow migrating bands of all the *P. penneri* LPSs, which correspond to high-molecular-mass LPS species consisting of the core-lipid A moiety with the attached long-chain O-specific polysaccharide. These results give evidence that the cross-reactive LPSs and the homologous ones are identical.

However, after the absorption of the antisera to *P. penneri* 107, 52, 34, 8, and 103 with cross-reacting antigens, the sera still reacted with the homologous LPSs up to titers of 1:400 (antiserum to *P. penneri* 103) and 1:3200–1:6400 (the remaining four antisera). The results suggest that in these five *Proteus* serogroups (O8, O61, O65, O67, and O73) there are some differences in strains epitope specificity. Banding patterns of Western blot assays indicate that the differences in LPS molecules from the O8, O61, O65, and O67 serogroups, responsible for the incomplete absorption of the antisera against these four serogroups, are located in the core regions of the LPS molecules (Fig. 1). Fast-migrating LPS species containing the lipid A region and core oligosaccharide showed weaker or no reactions with the O-antisera for O8 (*P. penneri* 112 LPS), O61 (*P. penneri* 104, 133 LPSs), O65 (*P. penneri* 77, 78, 80, 81, 84–86, 88, 89, 95, 129 LPSs), and O67 (*P. penneri* 93 LPS). These results explain why after the absorption of the four antisera with the respective antigens mentioned above, some fractions of antibodies, reacting with the homologous LPSs, were left (see Table 2, column 7). These fractions are most probably antibodies recognizing LPS core epitope(s). This suggestion is also confirmed by the fact that the antisera against *P. penneri* 34 and *P. penneri* 8 without anti-core antibodies (removed by absorption with LPSs containing the same core regions but different O-parts) did not react in the homologous system after absorption with LPSs from *P. penneri* 77, 78, 80, 81, 84–86, 88, 89, 95, 129, and 93 (data not shown).

These results are in good agreement with our knowledge about a typical feature of the LPS core region in *Proteus* rods, namely their structural variability from strain to strain and even within a strain[23]. The structure of the core region can vary not only in LPSs from different O-serogroups, but also within one O--serogroup are *Proteus* strains containing different types of LPS core. In this way it can happen that *Proteus* strains from different O-serogroups have the same LPS-core structure (R-antigen)[22]. However, the differences in LPS core region do not influence the serological classification of *Proteus* strains into O--serogroups, based on the composition of O-antigens.

In the case of *P. penneri* 103 antiserum, although the absorption of this antiserum with cross-reacting *P.*

Table 2. Serological cross-reactions of investigated *P. penneri* LPSs with the respective O-antisera

No.	Serogroup	Antiserum to strain	LPS from investigated *P. penneri* strains[a]	Reciprocal titer for		Minimal dose (ng) of LPS inhibiting the homologous test system in		Reciprocal titer for PIH in homologous system after absorption of antiserum
				PIH	EIA	PIH	EIA	
1	**2**	**3**	**4**	**5**		**6**		**7**
1.	O8	*P. penneri* 107	**107**, 106, 112, *P.v.* O8	25 600–51 200	128 000	1	2–4	<100 (1 600)*
2.	O17	*P. penneri* 16	**16**, 108, 111, 131	51 200	128 000–512 000	4–8	4–8	<100
3.	O31	*P. penneri* 28	**28**, 46, 126, 134	25 600–51 200	256 000–512 000	2–8	8–16	<100
4.	O52	*P. penneri* 15	**15**, 49, 76, 91, 110, 130	25 600–102 400	128 000–1 024 000	4–8	4–16	<100
5.	O58	*P. penneri* 11	**11**, 115, 125	25 600	512 000	0.5	1	<100
6.	O61	*P. penneri* 52	**52**, 92, 104, 109, 116, 118, 127, 133, 136	25 600–102 400	128 000–512 000	0.5–8	1–4	<100 (3 200)*
7.	O62	*P. penneri* 41	**41**, 102, 113	51 200	156 000	0.5–4	1–2	<100
8.	O64a	*P. penneri* 19	**19**, 82, 83, 87, 94, 96, 100, 105, 114, 120, 122	51 200–102 400	128 000–512 000	2–4	1–2	<100
9.	O65	*P. penneri* 34	**34**, 77, 78, 79, 80, 81, 84, 85, 86, 88, 89, 95, 117, 119, 129	51 200–102 400	128 000–512 000	0.5–4	0.5–4	<100 (6 400)*
10.	O67	*P. penneri* 8	**8**, 93, 101	51 200–102 400	128 000–256 000	8	2	<100 (6 400)*
11.	O69	*P. penneri* 25	**25**, 121, 123	12 800	128 000–256 000	1–2	1–2	<100
12.	O73	*P. penneri* 103	**103**, 90, 128	51 200–102 400	128 000–256 000	1–2	1–2	<100 (400)*

[a] The homologous LPSs are bolded.

* Results for antigens differing in epitope specificity from the homologous ones.

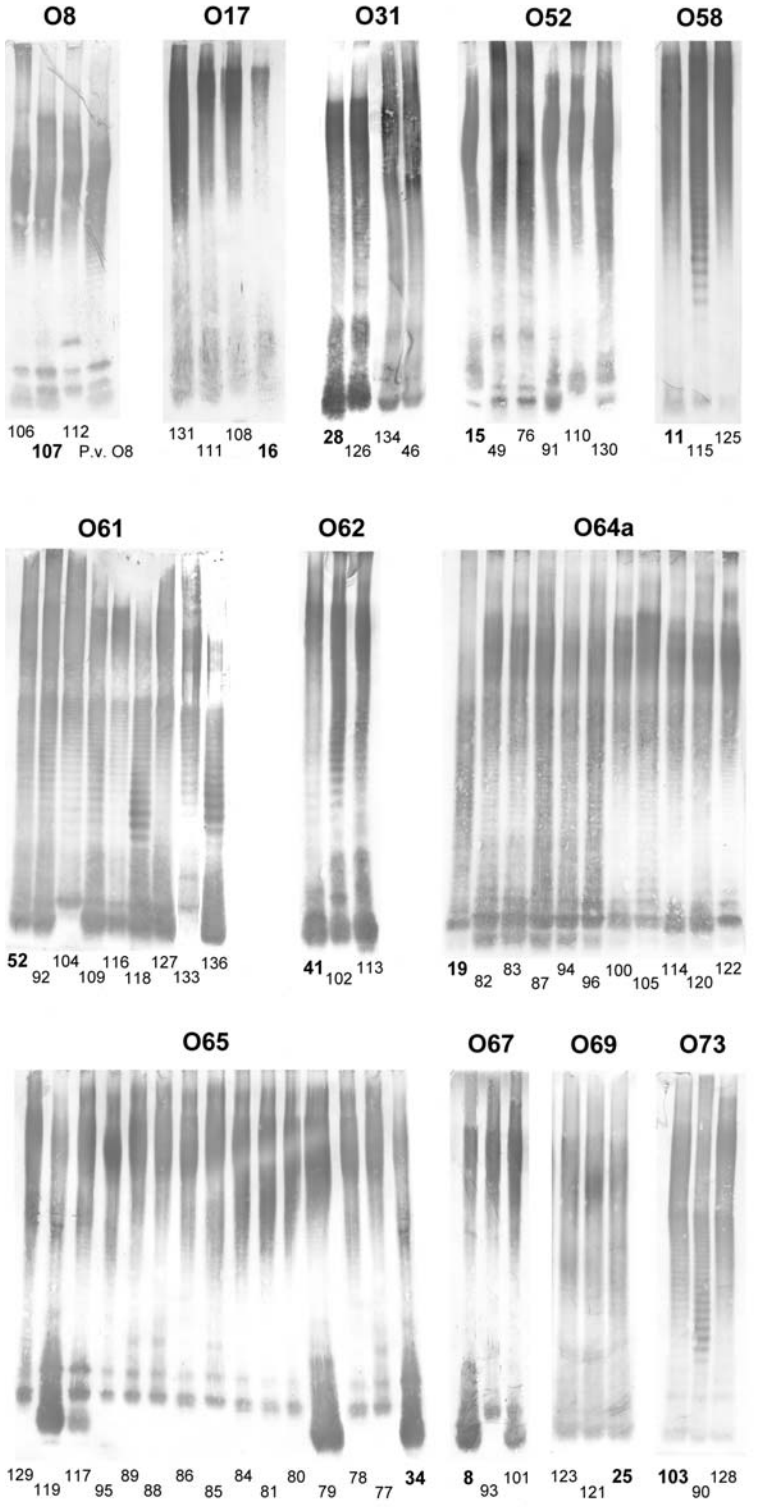

Figure 1. Reactivity of the antisera against *Proteus* strains, representing particular *Proteus* O serogroups, with LPSs of *P. penneri* strains studied. P. v. stands for *P. vulgaris*. The LPSs homologous to the used antisera are bolded.

penneri 90 and 128 LPSs left a small fraction of antibodies (Table 2, column 7), there are no visible differences in Western blot analysis, and all the LPSs reacted strongly both in the O-specific polysaccharide-containing and in lipid A – core LPS molecules (Fig. 1). There are probably small epitope-specificity differences between these LPSs. This problem will be resolved in further investigations, including studies of the chemical structures of these O antigens. The two cross-reacting strains *P. penneri* 90 and 128 are classi-

fied within *Proteus* serogroup O73 together with *P. penneri* 103.

As a result of these investigations, all the *P. penneri* strains studied (57) were classified into 13 *Proteus* O serogroups. Figure 2 illustrates the participation of the strains collection in the particular O serogroups. The largest number of strains (14) belongs to *Proteus* serogroup O65, which was represented only by one *P. penneri* strain 34 before[27]. As many as 11 of these strains were isolated in Braunschweig (Germany) from the stools of patients and healthy people. These data indicate that the *Proteus* O65 serogroup is widespread in this area. One can suggest that the O antigen chemical structure of this serotype does not contribute to the virulence of the bacteria because the strains were isolated from patients with gastroenteritis as well as from healthy volunteers. The *P. penneri* 34 (O65) O antigen repeating unit carries equal numbers of positively and negatively charged groups[22], a feature which is considered important for the adaptation of *Proteus* bacilli to grow under different pH conditions[8] and which may be one of the reasons for the frequent isolation of strains from this serogroup in the area mentioned.

In summary, 16 isolates were investigated and classified into serogroups O8, O17, O31, O52, and O69, composed of two, three or four *Proteus* species (O8 – *P. mirabilis, P. vulgaris, P. penneri, P. genomospecies* 5; O17 – *P. mirabilis, P. vulgaris, P. penneri*; O31 – *P. vulgaris, P. penneri*; O52 – *P. vulgaris, P. penneri, P. hauseri*; and O69 – *P. mirabilis, P. vulgaris, P. penneri, P. genomospecies* 6) (ref.[7] and Sidorczyk, unpublished data). However, strains from these serogroups are not frequently isolated from sick persons (ref.[11] and Sidorczyk, unpublished data). It is important to emphasize that 72% of the studied strains (41) were classified into serogroups that contain *P. penneri* strains only. Such results confirm the diversity of this species among the genus *Proteus*, which has also been proved using genetic, biochemical, and serological methods[3, 6, 10, 27] and may have diagnostic significance.

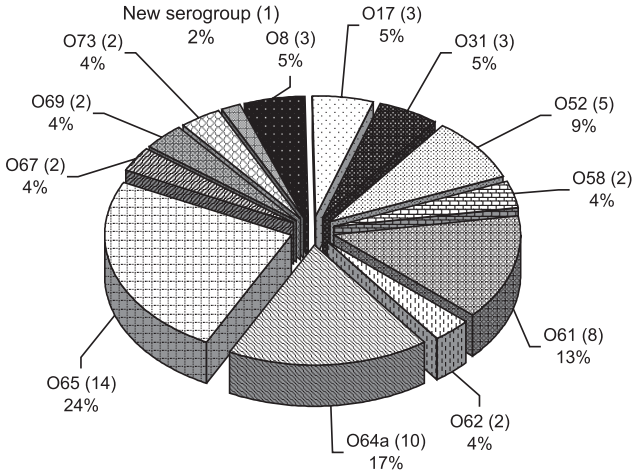

Figure 2. The contribution of studied 57 *P. penneri* strains in particular *Proteus* O serogroups. In parenthesis the number of strains is given.

Among the 57 *P. penneri* strains of the collection studied, only one (*P. penneri* 97) created a new *Proteus* O serogroup, and two (*P. penneri* 90 and 128) were classified into a new *Proteus* O73 serogroup established very recently[4]. It is worth mentioning that three strains (*P. penneri* 106, 107, and 112) belong to *Proteus* serogroup O8, which up to now has not contained any *P. penneri* strains. The remaining 51 *P. penneri* strains (89% of the studied collection) were classified into *Proteus* O serogroups established before.

The results obtained in this work indicate that the studies aimed at the serological classification of *Proteus* bacilli with regard to *P. penneri* are approaching a successful end. Although the number of new *P. penneri* isolates from patients grows, the number of new *Proteus* serogroups practically does not increase, because most new *P. penneri* clinical strains belong to the already existing *Proteus* O serogroups.

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