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The use of the PhP-KE biochemical fingerprinting system in epidemiological studies of faecal *Enterobacter cloacae* strains from infants in Swedish neonatal wards

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SUMMARY

The PhenePlate (PhP) biochemical fingerprinting system is an automated method for typing of bacteria, based on the evaluation of the kinetics of biochemical reactions, performed in microtitre plates. In the present study the PhP-Klebsiella/Enterobacter (KE) system was evaluated for typing of *Enterobacter cloacae* and employed to study the epidemiology of faecal *E. cloacae* strains isolated from infants in 22 Swedish neonatal wards. The PhP-KE system showed a high reproducibility and discrimination for *E. cloacae* isolates. Among 64 epidemiologically unrelated *E. cloacae* strains, 49 distinct phenotypes were found, and the diversity index was 0·985. *E. cloacae* was found as a part of the dominating Gram-negative aerobic bacterial flora in 83 out of 953 infants studied. The incidences of *E. cloacae* colonization varied between 0 and 35% in different wards, but in contrast to previous data for *Klebsiella* spp. and *Escherichia coli*, there was little evidence of spread of particular strains in the wards. We also discuss two different measures of nosocomial transmission of bacterial strains: transmissible strains and epidemic index.

INTRODUCTION

Gram-negative bacteria continue to be a major cause of hospital-acquired infections. The proportion of *Enterobacter* spp. in such infections seems to remain constant (about 10% in US hospitals) [1]. In addition to the sporadic cases normally occurring, many nosocomial *Enterobacter* spp. outbreaks have been described, e.g. in neonatal intensive care units [2–5]. In recent years, *E. cloacae* has received worldwide attention due to its high propensity for developing mutational resistance to most old and new beta-lactam antibiotics, also at the site of infection during therapy [6]. Due to the lack of generally available typing methods, the nosocomial epidemiology of *Enterobacter* spp. is incompletely understood [7].

We have studied aerobic, Gram-negative faecal bacteria, collected from 953 infants in 22 Swedish neonatal wards during 1984–5 [8]. The most common species

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found were *Klebsiella oxytoca* (69% of the neonates colonized) and *Escherichia coli* (44%). These isolates were further characterized, using a recently developed automated system for typing of bacteria, based on the evaluation of the kinetics of a set of biochemical reactions, performed in microtitre plates (the PhenePlate or PhP system) [9, 10]. Among about 1000 distinct *E. coli* and *Klebsiella* spp. phenotypes identified, we found about 20 major (M-)phenotypes (transmissible phenotypes), some of which had colonized up to 50% of the neonates in certain wards [8].

After *K. oxytoca* and *E. coli*, *E. cloacae* was the next most common species isolated. In the present investigation, the PhP typing system was evaluated for typing of *E. cloacae*, and then used to study the epidemiology of faecal strains of this species in the neonatal wards mentioned above.

MATERIAL AND METHODS

The PhenePlate Klebsiella/Enterobacter *system*

The PhP typing system for *Klebsiella* and *Enterobacter* spp. (PhP-KE system) consists of three identical sets of 32 dehydrated reagents in a prefabricated microplate (BioSys inova, Stockholm) (Table 1). The reagents have been specially selected to yield a high discrimination among isolates belonging to *Klebsiella* and *Enterobacter* spp. [10] One colony of the isolate to be tested was suspended into 10 ml of a medium containing 0·05% proteose peptone (Difco) and 0·01% bromothymol blue, and 0·15 ml of the suspension was added to each one of the 32 wells. The microplates were incubated at 37 °C and read after 7, 24 and 48 h using a microplate reader connected to a personal computer [10]. The mean absorbance value of each reaction was calculated after the final reading, yielding a biochemical fingerprint of each isolate consisting of 32 numbers. The fingerprints of all tested isolates were compared pairwise, and their similarities were calculated and expressed as correlation coefficients. The correlation matrix thus obtained was clustered according to the UPGMA method [11], yielding a dendrogram. All handling of optical readings and calculations including clustering and printing of dendrograms was performed using the PhP software (BioSys inova, Stockholm).

Reproducibility and identity level

The reproducibility of the PhP-KE system for *E. cloacae* was measured by calculating the mean of correlation coefficients obtained from duplicate assays of 22 distinct strains. The minimum degree of similarity between fingerprints of different isolates required to define identity (the ID level) was defined as the mean correlation coefficient for duplicate assays minus $2 \times SD$ (95% confidence level) [10]. Isolates showing correlation coefficients equal to, or higher than, the ID level when compared were thus assigned to the same biochemical phenotype.

Definition of phenotypes

Biochemical phenotypes which were found in more than one infant in the present study were named C(Common)-phenotypes, and phenotypes found in a minimum of 10% of the infants in a particular ward were defined as M(major)-

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Table 1. *Reagent panel used in the PhP-KE system*

Test no.	Reagent	Test no.	Reagent
1	Cellobiose	17	Sorbose
2	Lactose	18	Deoxyglucose
3	Raffinose	19	Deoxyribose
4	D-Arabitol	20	L-Fucose
5	Sorbitol	21	D-Fucose
6	Rhamnose	22	Dulcitol
7	Arbutin	23	Tagatose
8	Salicin	24	Gluconate
9	D-Arabinose	25	Citrate
10	Palatinose	26	Malinate
11	Lactulose	27	Malonate
12	Melezitose	28	L-Tartrate
13	Adonitol	29	Ornithine
14	Glycerol	30	Urea
15	Inositol	31	Galactolactone
16	Maltitol	32	Negative control

phenotypes. The term S(Single/Sporadic)-phenotypes was used to define phenotypes which only occurred once.

Discrimination power

The discrimination power of the PhP-KE system was calculated in the following ways.

Resolution index (1 minus the mean correlation coefficient for a studied population of isolates). This index measures the metabolic diversity of the strains and thus degree of differentiation between phenotypes obtained using a particular set of characters. The resolution index can be used for selecting an optimal set of characters, since it will decrease if characters which do not discriminate between the phenotypes are included in the set [10, 12].

Accuracy index (1 minus the proportion of correlation coefficients in the interval between the ID level and the ID level minus $1 \times$ SD). This index shows the proportion of correlation coefficients close below the ID level, and is a measure of how often a pairwise comparison between two strains will yield a correlation coefficient making them very similar, but not identical [12].

Diversity index. Simpson's index of diversity (D_i) was calculated as

$$D_i = 1 - \Sigma(N_i(N_i - 1))/(N(N-1)),$$

where N_i equals the number of isolates found within each phenotype and N equals the total number of isolates assayed. It represents the probability that two random strains will indeed be assigned to different phenotypes [13]. Thus, for a given number of phenotypes found in a population, D_i is higher when the isolates are evenly distributed among all phenotypes than if many isolates fall into a few dominating phenotypes.

The above measures of discrimination power have been discussed recently [12].

Epidemic index

The epidemic index (E_i) was constructed in order to quantify the repeated occurrence of phenotypes in a sample of epidemiological interest (e.g. a population

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of humans, or in different wards). It was calculated in a similar manner to Simpson's diversity index (D_i) as

$$E_i = \Sigma(N_i(N_i-1))/(N(N-1)),$$

where N_i equals the number of isolates within each phenotype and N equals the number of individuals in the studied population, i.e. in the present study the number of infants in each ward. A low E_i for a particular ward here indicated that the phenotypes were randomly distributed among the infants in the ward, and a high E_i indicated that a large proportion of infants in the ward carried strains belonging to a few, dominating phenotypes.

Bacterial isolates studied

The 22 neonatal intensive care wards studied were followed for a mean period of 4 months. On the day of discharge, rectal swabs were taken from all infants with a minimum stay in the ward of 5 days ($n = 953$). From each faecal specimen, five Gram-negative isolates were collected [8], and identified by standard methods. Two *Enterobacter* spp. colonies from each sample were further analysed. *E. cloacae* was isolated from 83 infants altogether out of the 953 studied, yielding 166 isolates to be typed by the PhP-KE system.

Two different *E. cloacae* phenotypes were found in each of five infants, and one phenotype in each of the others. Thus, 88 different *E. cloacae* strains were available for studies of the epidemiology of faecal *E. cloacae* phenotypes in the 22 neonatal wards. The species identification of these strains was confirmed by the API 20E system (La Balme les Grottes, France).

RESULTS

Methodological aspects

Using the PhP-KE system, the mean correlation coefficient obtained from duplicate assays of 22 *E. cloacae* isolates was 0·989, with a standard deviation of 0·007, resulting in an ID level of 0·975. The accuracy index was 0·994, i.e. in only 6 out of 1000 cases a pair of strains yielded correlation coefficients just below the ID level.

The biochemical fingerprints of the 88 distinct *E. cloacae* strains isolated were compared within each neonatal ward, using the above ID level. Isolates from the same ward, and belonging to the same biochemical phenotypes, were regarded as epidemiologically related, and therefore only one isolate from each phenotype within the ward was selected for the assay of discrimination power. This resulted in 64 distinct strains.

The resolution index for these 64 strains was 0·334, indicating a high metabolic diversity among *E. cloacae* strains, and that the set of reagents used in the PhP-KE system was suitable for *E. cloacae*. The 64 distinct isolates yielded 50 different biochemical phenotypes, and a diversity index of 0·985. Thus, the discrimination power was satisfactory for *E. cloacae*, which indicates that this species is sufficiently heterogeneous in its biochemical reactions to be typed by the PhP system.

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Table 2. *Occurrence of* E. cloacae (*ECL*) *phenotypes in 22 Swedish neonatal wards*

Ward	No. of infants studied	No. of infants with ECL	No. of ECL strains	Distribution of phenotypes*	Epidemic index	Diversity index
UD	111	8	8	C5(3) 5S	0·001	0·893
OR	102	7	8	C16(2) 6S	0·000	0·964
JO	75	6	6	C15(2) C2(2) 2S	0·001	0·867
HE	74	26	28	M10(7) C8(4) C6(3) C7(2) 12S	0·012	0·922
VA	61	7	8	C14(2) 6S	0·001	0·972
KA	51	3	3	C12(2) 1S	0·001	0·667
MA	46	9	10	C11(3) C17(2) C9(2) S3	0·005	0·889
FA	43	1	1	1S	—	—
KK	41	1	1	1S	—	—
ES	35	3	3	C3(2) 1S	0·002	—
HU	35	1	1	1S	—	—
VX	33	5	5	C4(2) 3S	0·002	0·900
NO	18	2	2	2S	0·000	—
VK	17	1	1	1S	—	—
HA	12	2	2	2S	0·000	—
AN	11	1	1	1S	—	—
Other	188	0	—	—	—	—
Total	953	83	88		0·000	0·979

* C or M, phenotypes found in more than one infant in that particular ward. Numbers in brackets indicate the number of strains in each C- or M-phenotype. S, number of single phenotypes found in only one infant in that particular ward.

Epidemiological aspects

Of the 953 infants studied, *E. cloacae* was found in 9%. In 16 of the 22 neonatal wards, *E. cloacae* was found. The local incidences of infants colonized with *E. cloacae* ranged between 0 and 35% in different wards (Table 2), and the number of distinct phenotypes was between 0 and 16 per ward. Nevertheless, local spread of *E. cloacae* phenotypes seemed to be quite low, even in wards with high carrier rates. In nine wards, phenotypes which had colonized 2–7 infants in that particular ward were found (Table 2). A transmissible strain (M-phenotype) colonizing 10% of the infants was found only in one ward (ward HE, see Table 2).

Similarly, the local epidemic indices were generally low (always below 0·02). Thus, even the ward where 35% of the infants were colonized with *E. cloacae* showed an epidemic index of only 0·012 (ward HE, see Table 2).

Cluster analysis of faecal isolates of E. cloacae *from neonates*

The dendrogram derived from the correlation matrix obtained from pairwise comparisons of all 88 *E. cloacae* strains is shown in Figure 1. According to the PhP-KE system, the 88 isolates represented 50 distinct biochemical phenotypes. All faecal *E. cloacae* strains from the neonates, except for two, were distributed into two separate groups of phenotypes.

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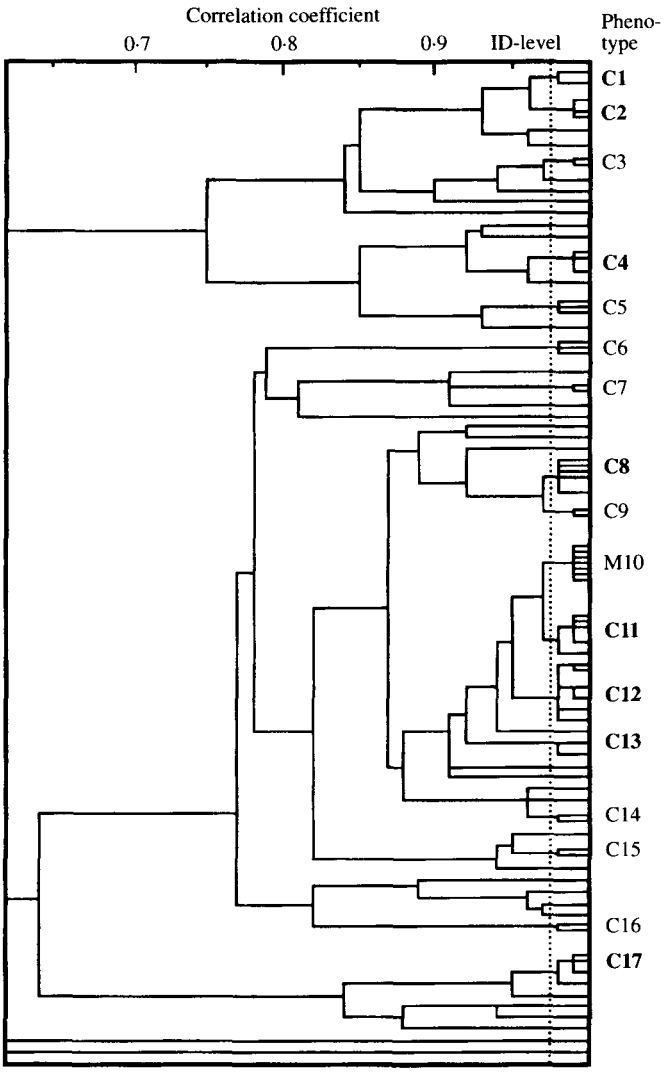

Fig. 1. Dendrogram from UPGMA clustering of the correlation matrix obtained from comparisons of 88 *E. cloacae* strains isolated from infants in neonatal wards. The identity level at a correlation coefficient of 0·975 is marked with a dotted line in the dendrogram. Strains which were found more than once within the total study are marked as C or M. Bold C indicates phenotypes which were found in more than one ward.

In the total material, 17 small clusters of identical strains which had colonized 2–7 infants (C-phenotypes) were found. Such strains had often been isolated from infants in the same ward, and within a short period of time (maximum 2 weeks), indicating nosocomial transmission. However, eight C-phenotypes were found in infants in two or more wards (C-phenotypes 1, 2, 4, 8, 11, 12, 13, and 17, see Fig. 1), and one of these (C12) occurred in as many as five different wards. The phenotype showing the highest overall prevalence in this material (M10) was the M-phenotype mentioned above. This phenotype was specific for ward HE, and five

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of the seven strains belonging to that particular phenotype had been isolated from infants cared for during the same time period.

DISCUSSION

Several typing methods have been used in epidemiological studies of *E. cloacae*. An O antigen serotyping scheme using 28 antisera was developed by Gaston and colleagues [14]. With these sera, 78% of 300 clinical isolates were typable, but 62% of the isolates belonged to the eight most frequent serotypes. A phage typing system comprising 25 phages yielded a high discrimination among the most common O groups and among 79% of the non-O typable isolates [15]. However, only 67% of the *E. cloacae* isolates retained the same lytic pattern after prolonged storage, and it was recommended that all isolates should be typed immediately [7]. Other conventional typing methods for *E. cloacae* are bacteriocin typing [16], and H antigen typing [17]. Advantages and disadvantages of these methods were recently discussed by Gaston [7]. Electrophoretic typing using isoenzymes has proved to give a good discrimination [18]. Electrophoretic methods based on whole-cell proteins [19], or on other cell constituents such as chromosomal or plasmid DNA may be highly discriminatory typing methods for *E. cloacae*, especially useful for studying small numbers of isolates. For large investigations, difficulties in obtaining a high degree of standardization between different assays may arise [18]. A biotyping scheme using seven substrates, developed by Old [20], discriminated 13 types among 110 *E. cloacae* isolates. However, Gaston and colleagues concluded that since 80% of *E. cloacae* isolates belonged to the two most common biotypes, this biotyping scheme is useful only for studies of the epidemiology of a strain with an unusual biotype [21]. Biotyping with the API 20E used by Gaston and colleagues [14] yielded 15 profiles among 327 clinical isolates, and 79% of these had the same API profile.

The PhP-KE system is also based on biochemical reactions of *E. cloacae* strains. We found the metabolic diversity of *E. cloacae* to be considerable, using the specially selected 32 biochemical reactions included in the PhP-KE system. This is reflected by the resolution index (0·334), a value similar to that which was earlier found for *Klebsiella* spp. [10] and *E. coli* [12]. The PhP system is very simple to manage using the pre-fabricated microplates, and one person may easily assay over 100 isolates in one day. All reactions may be read optically, and with the specially developed software the readings are directly transferred to a micro-computer, which automatically performs the calculations and produces and presents the typing result. Thus, only objective judgements of test results are involved, and due to the high interassay reproducibility of the PhP system, the biochemical fingerprints from different assays may also be compared.

The PhP-KE system may also be used for typing of *Klebsiella* spp. [10] as well as for other *Enterobacter* spp. and related species, e.g. *Serratia* spp. (unpublished data), using the same set of reagents and the same methods.

The stability of the biochemical phenotypes of *E. cloacae* over time has not yet been studied. However, we found several examples of presumed transmission of the same phenotype between the infants in neonatal wards, indicating that

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biochemical fingerprinting with the PhP-KE system is suitable for local studies of endemic and epidemic strains of *E. cloacae* over periods of at least a few months. The diversity indices for *E. cloacae* at the ward level were always higher than for the whole population of *E. cloacae* strains (Table 2). This indicates that the occurrence of certain C-phenotypes in the wards does not merely reflect a random distribution of *E. cloacae* strains, but rather a true transmission of a strain between the infants.

We have used two different terms to describe epidemic spread of bacterial strains, namely M-strains and epidemic index. These two terms measure different parameters. M-strains (Major strains) designate transmissible strains which are frequently found in the individuals of a studied population, e.g. in a ward. The epidemic index measures the distribution of different phenotypes within a population. A high incidence of individuals colonized by M-strains together with a low epidemic index indicates that the population has been colonized by many different transmissible strains, which in turn might be due to factors in the environment which favours spread of bacterial strains. A high incidence of M-strains together with a high epidemic index on the other hand, might be due to the occurrence of one or a few strains with a high ability to survive and spread in the population.

Nosocomial transmission of *E. cloacae* does not seem to be very common. Analyzed outbreaks of *E. cloacae* infections in hospitalized patients were usually found to be due to multiple strains [2, 7, 4, 22]. Flynn and colleagues concluded that *E. cloacae* from the patient's endogenous flora may cause infections, partly due to antibiotic selection, but that the strains are seldom transmitted between patients in a ward [23]. Similarly, in the 22 neonatal wards studied here during endemic periods, we could not verify any major outbreak of nosocomial transmission of *E. cloacae* strains. Fifteen small clusters of 2–7 infants carrying the same phenotype of *E. cloacae* were found in nine wards, but only in one ward a phenotype which had colonized 10% or more of the infants, and thus corresponds to our minimum definition of an M-strain [8], occurred. Also the epidemic indices for *E. cloacae* were generally low in all wards. The fact that some of the wards showed a relatively high incidence of infants colonized with *E. cloacae* therefore did not seem to be due to frequent local spread of certain strains. This is in contrast to our data for *K. oxytoca* and *E. coli* isolates from the same wards, showing several cases of wide nosocomial spread of certain phenotypes [8], and epidemic indices above 0·20 in some wards (to be published). The reason for these interspecies differences is not yet understood. Possibly the M-strains belonging to *E. coli* and *K. oxytoca* carry properties promoting their survival and spread in hospital environments, whereas *E. cloacae* lack such properties. A high occurrence of *E. cloacae* in a neonatal ward may then merely be due to the fact that it is continuously introduced into the hospital environment from an outside source which is particular to that area where the hospital is situated, e.g. the tap water.

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