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ORIGINAL PAPER

Usefulness of PCR Melting Profile Method for Genotyping Analysis of *Klebsiella oxytoca* Isolates from Patients of a Single Hospital Unit

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Abstract

The development of rapid and simple typing methods is required in order to identify possible sources of human exposure to opportunistic pathogens. *Klebsiella* spp. belongs to a group of bacteria that are opportunistic pathogens responsible for an increasing number of multi-resistant infections in hospitals. Recently, we showed the high genetic diversity of *K. oxytoca* using a large collection of strains isolated from the patients of several hospitals in Poland over a 50-year period. Our results showed that the internal transcribed spacer polymerase chain reaction method (ITS-PCR) is useful for the phylogenetic delineation of genetic groups in *K. oxytoca* and the high discriminatory power of the PCR melting profiles (PCR MP) method can be useful for epidemiological studies of *K. oxytoca*. In the present study the usefulness of PCR MP was tested on two sets of strains isolated from a single unit over a short period of time. The results revealed that PCR MP has a high discriminatory power and can be useful for epidemiological studies of closely related strains of *K. oxytoca* isolated from a single unit over a short period of time to identify the source, reservoirs and the tract of infection spread. The advantage of PCR MP for the above application was shown by using the procedure at increasing denaturation temperature during PCR to confirm genotyping results. Considering this feature and the high discriminatory power of PCR MP, as shown in this report for determination of the genetic similarities of consecutive *K. oxytoca* strains, we propose that PCR MP is one of the best techniques for short-term epidemiology analysis.

Key words: *Klebsiella oxytoca,* ITS-PCR, PCR MP, genotyping

Introduction

Klebsiella spp. embraces opportunistic pathogens that cause a wide spectrum of severe diseases such as septicaemia, pneumonia, urinary tract infection, and soft tissue infection. Nosocomial *Klebsiella* infections are caused mainly by *Klebsiella pneumoniae*. Since the early 1980s, isolates of *Klebsiella oxytoca* have been recognized as clinically significant and an indication for therapy (Livermore *et al.*, 1995). From year to year the number of infections caused by *K. oxytoca* has increased. Most reports regarding *K. oxytoca* have included epidemic cases that occurred in an outbreak (Garcia de la Torre *et al.*, 1985; Hansen *et al.*, 1988; Yinnon *et al.*, 1996; Watanakunakorn and Jura, 1991; Korvick *et al.*, 1992; Morgan *et al.*, 1984; Ransjo *et al.*, 1992). It is known that *K. pneumoniae* and *K. oxytoca* exhibit a high degree of genetic heterogeneity, as was demonstrated by capsular typing (Ørskov *et al.*, 1984), O-antigen variation (Mizuta *et al.*, 1983), biotyping (Rennie and Durcan, 1974), protein electrophoretic profiling (Ferragut *et al.*, 1989), multilocus enzyme electrophoresis (Combe *et al.*, 1994), ribotyping (Bingen *et al.*, 1993), randomly amplified polymorphic DNA (RAPD) analysis (Brisse and Verhoef, 2001), pulsed-field gel electrophoresis (Toldos *et al.*, 1997) and diversity of β-lactamase genes (Fournier *et al.*, 1996). Based on the sequence diversity of the *K. oxytoca* chromosomal β-lactamase and house-keeping genes, finally, six phylogenetic groups of *K. oxytoca* were determined (Fevre *et al*., 2005). In our previous study the diversity of *K. oxytoca* strains throughout over a 50-year period using internal transcribed spacer polymerase chain reaction (ITS-PCR) and PCR melting profiles (PCR MP) genotyping methods on a large collection of strains isolated from patients of several hospitals in Poland was analyzed retrospectively (Stojowska *et al*., 2009). Based on ITS-PCR method

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six phylogenetic groups of *K. oxytoca* was also determined. Typing by PCR MP method showed higher level of genetic diversity. However, all *K. oxytoca* strains were also divided into six distinct branches. We found that the ITS-PCR and PCR MP methods are useful for phylogenetic delineation of genetic groups in *K. oxytoca*.

The PCR MP method allows specific gradual amplification of genomic DNA in terms of thermal stability starting from less stable DNA fragments amplified at lower Td values to more stable ones amplified at higher Td values. Low Td during LM PCR leads to limited and specific amplification of a small number of less stable DNA fragments. The electrophoretic patterns of DNA fragments obtained after such amplifications are characteristic for the bacterial strain taken for DNA isolation. Using PCR MP we also have the possibility to increase the number of amplified DNA restriction fragments by increasing denaturation temperature during PCR. A steady increase in the number of amplified DNA fragments is dependent on denaturation temperature increase.

The objective of this study was to show the use of the PCR MP technique for routine epidemiological study of *K. oxytoca* strains isolated in a short period of time in individual hospital unit.

Experimental

Materials and Methods

Bacterial strains. The isolates included in the study were sent from the State Institute of Hygiene collection (Poland). The strain collection comprised 14 strains of *K. oxytoca* isolated during 15 years (1992–2007) from patients of Neonatal Intensive Care Unit of a hospital in Warsaw (set A of strains) and 14 strains isolated in 1999 from patients of Neonatal Intensive Care Unit of a hospital in Bydgoszcz (set B of strains).

Identification tests. All bacterial isolates were identified as *K. oxytoca* in the Department of Bacteriology at the National Institute of Public Health (Poland) by 40 non commercial classical biochemical tests. The final biochemical identification was carried out as described in Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994**)**. The methodological details of identification based on dulcitol and melezitose fermentation and sodium-potassium tartrate degradation were described previously (Kałużewski, 1967).

DNA isolation. DNA isolations (from a single colony on a Columbia sheep blood agar plate) were carried out with the DNA Genomic Mini kit (A&A Biotechnology, Poland) according to the manufacture's procedure with minor modifications. The DNA concentration ranged between 100 and 200 ng/µl (measured by NanoDrop 1000 spectrophotometer, Thermo Scientific).

Genotyping methods. ITS-PCR was performed according to the previously method described (Jensen *et al.*, 1993) with slight modifications. The primers (G1: 5'-GAAGTCGTAACAAGG-3' and L1: 5'-CAA GGCATCCACCGT-3') were designed based on the sequences complementary to the conserved regions of the 16S and 23S rRNA genes of various bacterial species. In a Biometra T-gradient thermal cycler, PCRs were performed as follows: 25 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min and elongation at 72°C for 1 min. Prior to cycling, 5 min denaturation step at 95°C was included. After the last cycle, samples were incubated for 5 min at 72°C. The amplification products were submitted to 4% polyacrylamide gels electrophoresis in TBE buffer. The gels were stained with ethidium bromide, visualized under an ultraviolet transilluminator and photographed using a Versa Doc Imaging System version 1000 (BioRad).

PCR MP was carried out according to the method described for *E. coli* isolates (Krawczyk *et al.*, 2006) with slight modifications. Digestion reactions were performed under uniform conditions using approximately 50 ng of DNA sample and 5 U of the HindIII endonuclease (Fermentas, Lithuania). Following a 30 min incubation at 37°C, ligation mix comprising 2 µl of two oligonucleotides forming an adaptor (POW, 5'-CTCACTCTCACCAACGTCGAC-3'; HINDLIG, 5'-AGCTGTCGACGTTGG-3'; 20 pmol each), 2.5 µl of ligation buffer (10 × concentrated; Epicentre, Madison, WI), 0.5 µl of 25 mM ATP (Epicentre), and 5U of T4 DNA ligase (Epicentre, Madison, WI) was added and the samples were incubated for 1 h at room temperature. Next, the mixture was heated in a thermoblock for 10 min at 70°C and cooled for 10 min at room temperature. The PCR was carried out in a 50 µl reaction mixture containing 1 µl ligation solution, 5 µl 10×PCR buffer (100 mM Tris-HCl, pH 8.5, 500 mM KCl, 1% Triton X-100), 2 µl 50 mM $MgCl_2$, 5 µl of a deoxynucleoside triphosphate mixture (concentration of each deoxynucleoside triphosphate, 2.5 mM), 2 U of Pwo polymerase (DNA Gdańsk II, Poland), and 50 pM of PowaAGCTT primer (5'-CTCACTCTCA CCAACGTCGACAGCTT-3'). In a Biometra T-gradient thermal cycler, PCRs were performed as follows: 7 min at 72°C to release unligated oligonucleotides HINDLIG and to fill in the single-stranded ends and create amplicons, followed by initial denaturation at 86°C for 90 s and 22 cycles of denaturation at 86°C for 1 min, and annealing and elongation at 72°C for 2 min. After the last cycle, samples were incubated for 5 min at 72°C. The denaturation temperature was calculated during the optimization experiments for several *K. oxytoca* isolates using a gradient

thermal cycler (Biometra Tgradient Engine) with a gradient range from 85 to 89°C for the denaturation step as described above. The amplification products were submitted to 6% polyacrylamide gels electrophoresis in TBE buffer. The gels were stained with ethidium bromide, visualized under an ultraviolet transilluminator and photographed using a Versa Doc Imaging System version 1000 (BioRad).

Fingerprint analysis and dendrogram constructions. The patterns obtained from the electropherograms were converted and analyzed using the Quanty One software, version 4.3.1 (Bio-Rad, USA). For ITS-PCR and PCR MP, the band positions in each gel were normalized using the M 100–1000 DNA ladder (DNA Gdansk, Poland). Band matching and isolate similarity was accomplished using Dice band-based coefficient of similarity, which provides the most accurate similarity results when compared with visual inspection of the fingerprint patterns (Carrico *et al.*, 2005). A dendrogram was constructed using the unweighted pair-group method with arithmetic averages (UPGMA), which employs a sequential clustering algorithm in which the relationships are identified in order of similarity and the dendrogram was built in a stepwise manner (Carrico *et al.*, 2005). The cut-off values for genotype definition were established as 95% and 90% for ITS-PCR and PCR MP, respectively.

Results

Genotyping of *K. oxytoca* strains isolated from a single clinical unit over 15-year period. ITS-PCR method was used to study genetic relationship between *K. oxytoca* strains, regarding to genetic diversity of *K. oxytoca* isolated in Poland over a 50-year period (Stojowska *et al.*, 2009). Five different amplification profiles at 40% of similarity level were identified by ITS-PCR for 14 clinical *K. oxytoca* strains (set A) isolated from a single clinical unit (Fig. 1; Table I). Clustering analysis of electrophoretic patterns groups the *K. oxytoca* strains into two major clusters, KoX1 and KoY1 (names of genotype groups and genotypes as in previous study, Stojowska *et al.*, 2009), suggest that the examined strains represent at least two different lineages. Low genetic diversity of *K. oxytoca* strains within groups (over than 75% of similarity) shows that these strains were probably closely related. One genotype group, KoX1, was markedly predominant, as this was represented by 12 clinical isolates (86%). The strains from KoX1 were classified as five different genotypes. One of them, KoX1-1, was predominant and was represented by 8 strains (57%). Two strains, which were classified as a group KoY1, show the same ITS-PCR amplification pattern (genotype KoY1-5). Strains of *K. oxytoca* which were classified to KoX1-1 genotype were identified in the whole period of time examined (from 1992 to 2007). The KoY1-1 genotype was identified twice only in 1995.

Genotyping by PCR MP showed high genetic diversity of *K. oxytoca* strains tested. As shown in Fig. 1 and Table I 13 different genotypes were distinguished, which were divided into four genotype groups: KoA (8 strains), KoC (2 strains), KoD (2 strains), and KoF (2 strains) at the cut-off level only 15% (names of genotype groups and genotypes as in previous study, Stojowska *et al.*, 2009). The absence of a predominant strain and high genetic diversity suggest that none of those strains is endemic in hospital.

Table I
Clinical data of colonized/infected patients in 15 years of study period. Names of ITS-PCR and PCR MP genotypes are related to our previous study (Stojowska *et al.*, 2009).

No	Date	Isolate source	PCR MP genotype group	ITS-PCR genotype
1	1992	stool	KoX1-1	KoA-1
2	1993	stool	KoX1-1	KoA-2
3	1995	stool	KoY1-5	KoF-1
4	1995	stool	KoY1-5	KoF-1
5	1996	stool	KoX1-1	KoA-3
6	1997	stool	KoX1-11	KoD-1
7	1999	stool	KoX1-5	KoC-2
8	1999	stool	KoX1-1	KoA-4
9	2000	throat	KoX1-1	KoA-5
10	2002	nose swabs	KoX1-1	KoA-6
11	2003	stool	KoX1-5	KoC-2
12	2005	stool	KoX1-1	KoA-7
13	2007	stool	KoX1-8	KoD-2
14	2007	stool	KoX1-1	KoA-8

Table II
Clinical data of colonized/infected patients in 1 year of study period. Names of ITS-PCR and PCR MP genotypes are related to our previous study (Stojowska *et al.*, 2009).

No	Patient	Isolate source	ITS-PCR genotype	PCR MP genotype
1	P1	stool	KoX1-1	KoA-1
2	P2	stool	KoX1-1	KoA-1
3	P3	throat	KoX1-1	KoA-1
4	P3	stool	KoX1-1	KoA-1
5	P4	throat	KoX1-1	KoA-1
6	P4	urine	KoX1-1	KoA-1
7	P4	pus	KoX1-1	KoA-1
8	P4	throat	KoX1-1	KoA-1
9	P4	stool	KoX1-1	KoA-1
10	P5	urine	KoX1-3	KoC-1
11	P6	urine	KoX1-3	KoC-1
12	P7	urine	KoX1-3	KoC-1
13	P8	urine	KoX1-3	KoC-1
14	Hospital environment	swab	KoX1-3	KoC-1

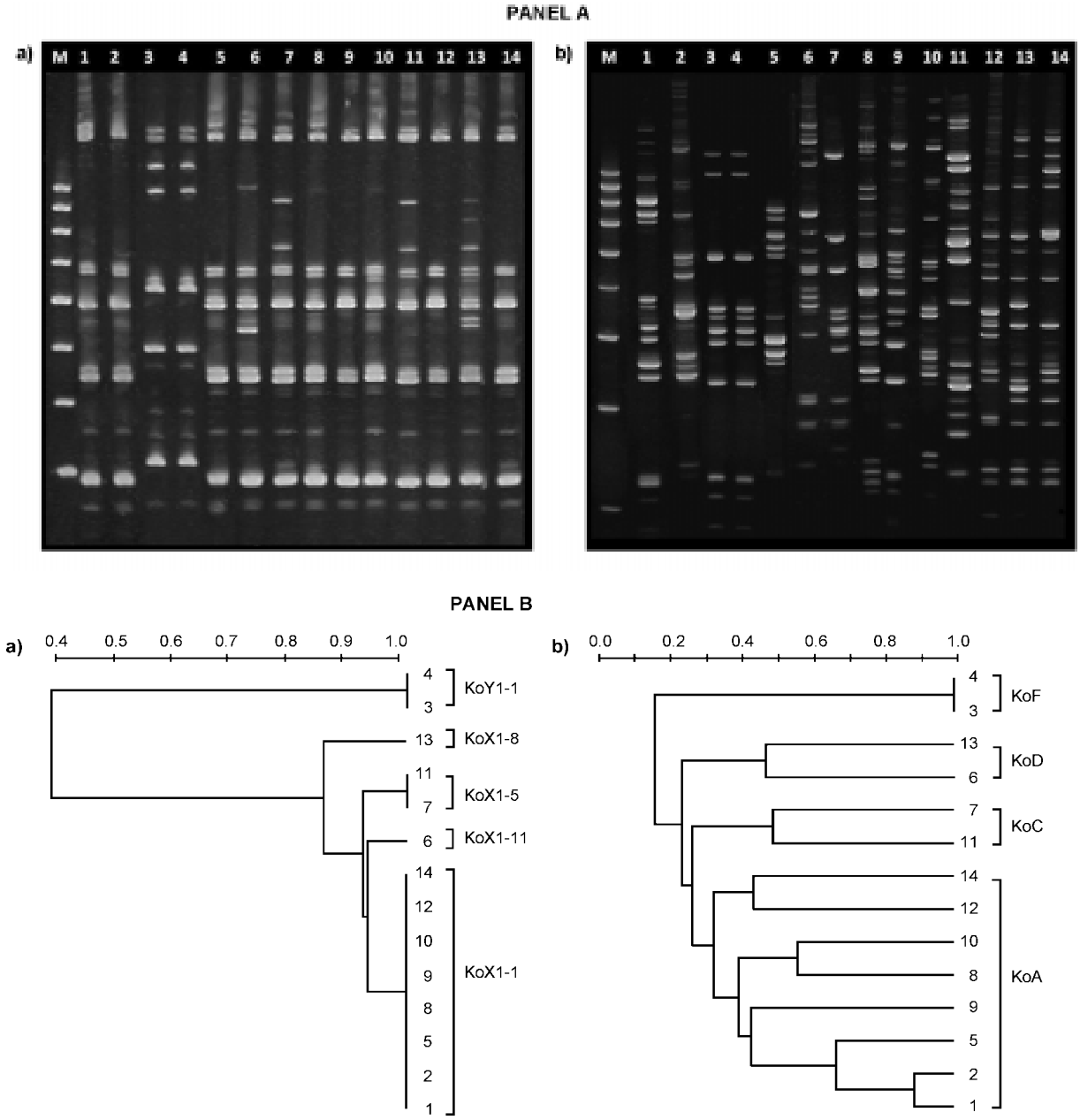

Fig. 1. Panel A: ITS – PCR (a) and PCR MP (b) electrophoresis patterns of *K. oxytoca* strains isolated from single clinical unit in Poland over a 15-year period. M – molecular size marker (100–1000 bp); from 1 to 14 – numbers of *K. oxytoca* strains. Panel B: Dendrogram of *K. oxytoca* strains based on ITS – PCR (a) and PCR MP (b) methods generated with Dice Coefficient (DC) and the UPGMA clustering method.

We found complete agreement between the grouping as defined by ITS-PCR and PCR MP. All strains from group KoA, KoC and KoD (typed by PCR MP) belong to KoX-1 group (typed by ITS-PCR) and two strains of KoF group belong to KoY group.

Genotyping of *K. oxytoca* strains isolated from a single clinical unit over 1-year period. The typing of *K. oxytoca* isolated from one single clinical unit revealed small genetic diversity among 14 strains (set B) isolated from single clinical unit over a 1-year period (Fig. 2). Typing by both ITS-PCR and PCR MP grouped all strains into two clusters (Table II). Each cluster grouped strains at 100% of similarity level (all strains in one cluster showed the same amplification pattern – the same genotype). Genotypes KoX1-1 and KoX1-3, distinguished by ITS-PCR showed 80% of similarity level and belonged to the same sub-group (KoX1). Genotypes KoA1-1 and KoC1-1 identified by PCR MP were not closely related (15% of similarity) and belonged to two different genotype groups. Clustering analysis based on ITS-PCR and PCR MP results indicates the presence of clonally dependent strains, which can be responsible for nosocomial infections. The existence of two

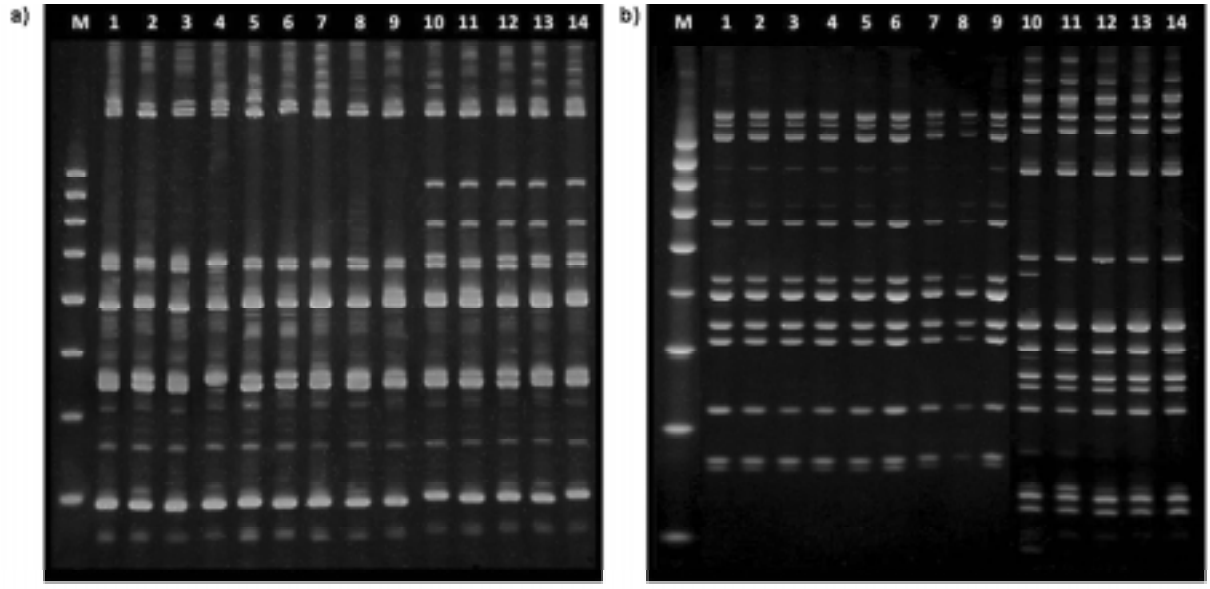

Fig. 2. ITS – PCR (a) and PCR MP (b) electrophoresis pattern of *K. oxytoca* strains isolated from single clinical unit in Poland.
M – molecular size marker (100–1000 bp); from 1 to 14 – numbers of *K. oxytoca* strains.

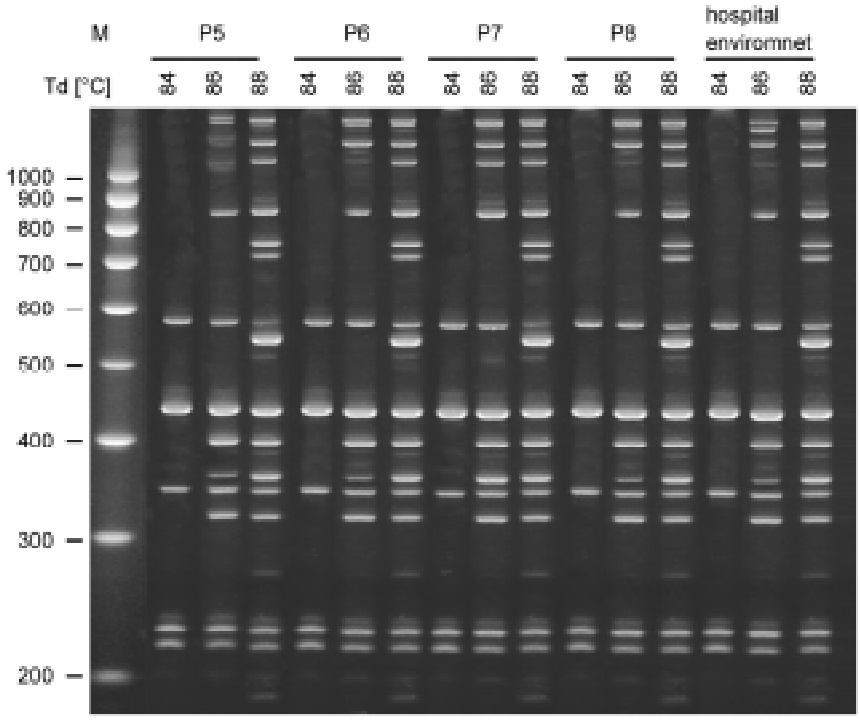

Fig. 3. PCR MP fingerprint at increasing denaturation temperatures (84°C, 86°C and 88°C) of *K. oxytoca* strains isolated from different patients and from the hospital environment.
M – molecular size marker (100–1000 bp); P5, P6, P7, P8 – numbers of patients.

different groups of genotypes probably indicates two different sources of infection in this clinical unit.

Typing by both ITS-PCR and PCR MP methods showed that *K. oxytoca* strain isolated from the hospital environment (the baby changing table) was classified into the same genotype as four other strains isolated from four patients (P5, P6, P7, P8) (Fig. 2; Table II). To ensure that those 5 isolates belonging to the same genotype PCR-MP at increasing denaturation temperatures were carried out. A steady increase in the number of amplified DNA fragments, which is dependent on Td increase, was observed and still produced identical profiles for isolates belonging to the same genotype (Fig. 3). Based on this experiment we

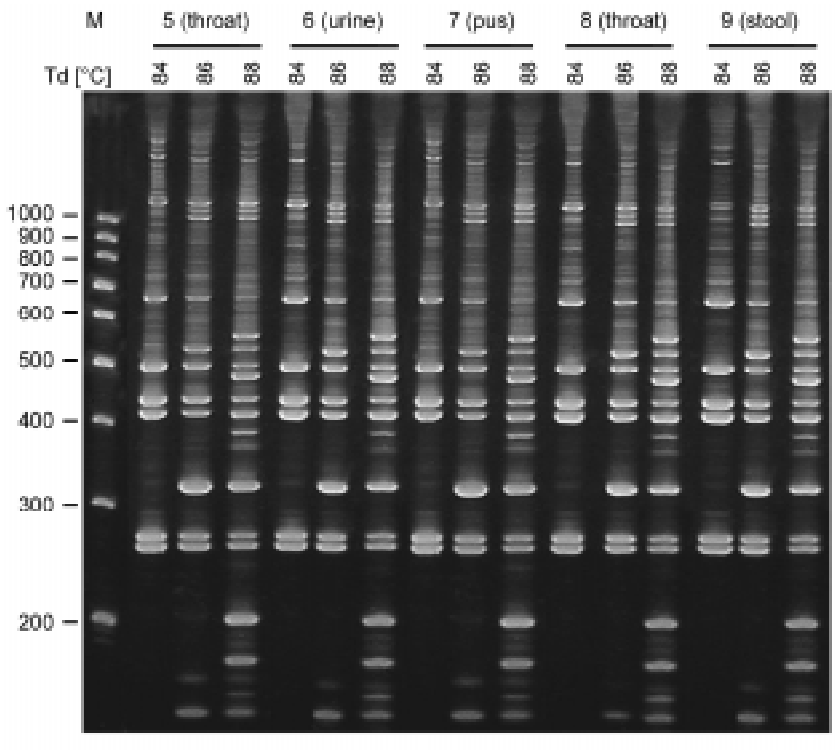

Fig. 4. PCR MP fingerprint at increasing denaturation temperatures (84°C, 86°C and 88°C) of *K. oxytoca* strains isolated from different sources of one patient.
M – molecular size marker (100–1000 bp); from 5 to 9 – numbers of *K. oxytoca* strains isolated from patient P4.

can conclude that those five isolates of *K. oxytoca* were probably clonally related and the hospital environment was the source of nosocomial infection.

Five *K. oxytoca* strains isolated from different sources of one patient (P4) were classified into the same genotype (both ITS-PCR and PCR MP) (Fig. 2, lines 5–9; Table II). The presence of clonally related strains in throat, urine, pus, faeces may indicate a spread of bacteria within patient. The PCR MP experiment at increasing denaturation temperatures (Fig. 4) confirmed that isolates suspected of playing a role in the spread of bacteria within patient were clonally related.

Discussion

Currently, nucleic acid-mediated techniques are more frequently applied and better appreciated than the phenotypically oriented approaches in taxonomy, epidemiology, and evolutionary studies. There are many different methods for determination of genetic variation among microbial isolates. Each of these methods has its technical and nucleic acid target dependent limitations, which should be taken into account when performing molecular typing studies and subsequently calculating strain relatedness. Furthermore, space and time need to be considered when selecting the optimal molecular markers. Small-scale epidemiological studies require different approaches in comparison to the analysis of a worldwide or nationwide spread of certain microbes. Actually, macrorestriction analysis of genomic DNA followed by pulsed field gel electrophoresis (REA-PFGE) is regarded as "the gold standard" for molecular typing of many microorganisms (Van Belkum *et al*., 1998). However, problems such as time consuming analyses, cost limitations, electrophoretical resolution or the need for special equipments still remain to be solved to expand the practice of bacterial typing at the strain level. In our study we used two different genotyping methods, ITS-PCR and PCR MP, for determination of genetic variety of *K. oxytoca* strains isolated from patients of two different clinical units over a 15-year and 1-year period, respectively.

Typing by ITS-PCR the clinical set A of *K. oxytoca* strains was divided into two groups of genotypes: KoX1 and KoY1. This suggests that the examined strains represented at least two different lineages. KoX1 group and KoX1-1 genotype were markedly predominant. This results confirmed our earlier results (Stojowska *et al*., 2009), where KoX1-1 strains of *K. oxytoca* were identified with the highest frequency during over 50-year period in Poland (since 1965).

Based on our experiments we can conclude that ITS-PCR in comparison to PCR MP has lower level

of discriminatory power and may be chosen to study phylogenetic delineation of genetic groups in *K. oxytoca*. The PCR MP method has a high discriminatory power and can be useful for epidemiological studies of closely related strains of *K. oxytoca* isolated from a single unit over a short period of time. This method is especially dedicated to identify of source, reservoirs, tract of infection spread and to distinguish between epidemic and endemic strains. The advantage of PCR MP for the above application is the possibility of increasing the number of amplified DNA restriction fragments by increasing denaturation temperature during PCR to confirm genotyping results.

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