ISSN 0233-7657. Біополімери і клітина. 2003. Т. 19. № 6

A PCR-mediated method for discrimination of *Klebsiella oxytoca* between closely related bacteria in environmental and clinical specimens

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A specific detection method was developed to discriminate Klebsiella oxytoca between other species of the genus Klebsiella on the basis of PCR amplification of the unique DNA sequences within the polygalacturonase-encoding (pehX) gene. Four primers have been designed for performing PCRs gaining amplicons of 282, 344, 451 and 513 bp. The specificity of the test was verified by the lack of PCR products in case of related K. pneumoniae, K. planticola, and polygalacturonate-degrading species of the genus Erwinia. The PCR-mediated test gives a rapid answer, concerning the presence of K. oxytoca in a sample, or in differentiating this bacterium from other species, such as K. pneumoniae, with which they can be confused. The diagnostic test can be used in ecological monitoring of K. oxytoca as well as in medical laboratories.

Introduction. The bacteria *K. oxytoca* and *K. pneumoniae* are controversial species of the genus *Klebsiella*. On the one hand, they are known as nitrogen-fixing organisms beneficial in agriculture [1]. Besides, they are concerned with pioneer work on genetics [2, 3] and enzymology of nitrogen (N_2) fixation [4]. The nitrogen-fixing *K. oxytoca* strains have been isolated from the rhizosphere of rice [5], or from the interior of rice and sweet potato roots [6, 7]. *K. pneumoniae* has been isolated from the interior of maize roots [8]. At the same time, representatives of these two species are described as causal agents of human diseases [9].

The taxonomic position of *K. oxytoca* is still not clear, this bacterium is often in «the shadow» of *K. pneumoniae* as it was considered for a long time as a subspecies of the latter [10], and was separated only according to the DNA-DNA hybridization and phenotype characteristics [11]. According to the worldwide predominant Orskov's classification [10] *K. oxytoca* is an independent species of the genus, however, within the classification of Cowan [9] which is also valid, there is no such a name. The lack of a consistent nomenclature results sometimes in mistakes in differentiation of the two species. Nitrogen-fixing isolates of *Klebsiella*, possessing distinguishing characteristics of *K. oxytoca*, have been designated as *K. pneumoniae* (for example, strain *K. pneumoniae* M5a1) [1]. Consequently, the name *K. pneumoniae* instead of *K. oxytoca* is probably used in many publications improperly. Nowadays, due to the rapid development of bioinformatics, DNA sequences of improperly named bacteria being deposited in public gene banks and spread quickly, determine the appearance of more errors.

Another reason for the misidentification of *K. oxytoca* is that detecting discriminative trait of *K. oxytoca* such as pectate degradation appears too labour-consuming to be taken into account in the identification process. The Biolog system and commercial biochemical tests (API 20E) designed for the *Enterobacteriaceae* identification are unreliable being used for the *Klebsiella* species differentiation. A new identification system designed on the basis of colony color and morphology on CHROMagar orientation

medium in combination with simple biochemical tests such as indole, lysine decarboxylase, and ornithine decarboxylase does not discriminate properly between *K. oxytoca* and *K. pneumoniae* as well [12].

Various DNA probes have been used in several hybridization and PCR procedures to distinguish *K. oxytoca* [13—18]. However, none of these techniques is completely satisfactory since the sequences used are not present exclusively in *K. oxytoca* (e. g. *gyrA*, *parC*, *infB*, *rrs*, and *bla*). This can lead to cross-reactions between the probe and DNA sequences from other species of the genus *Klebsiella* and from other enterobacteria resulting in a large number of false negatives or false positives.

The aim of this study was to devise a rapid and sensitive test for the discrimination between *K. oxytoca* and close relatives and detection of this bacterium in the environmental and clinical specimens. The idea was to exploit a pectate degradation capability of *K. oxytoca*, which distinguishes the bacterium from other species. More specifically, we used a unique sequence of the gene encoding the enzyme polygalacturonase that cleaves a polygalacturonic chain of demethoxylated pectin.

Materials and Methods. Bacteria are listed in Table 1.

DNA of nitrogen-fixing strain *K. pneumonia* 342 [8], *K. pneumonia* ZMVSY isolated from Wisconsin field, and clinic strain *K. pneumonia* MGH78578 were obtained from Dr. Eric Triplett (Wisconsin University).

Biochemical tests. Bacterial strains were considered as belonging to the genus *Klebsiella* on confident identification as «*K. oxytoca* or *K. pneumoniae*» after biochemical key tests such as indole (positive for *K. oxytoca*, negative for *K. pneumoniae*), melezitose (positive for some strains of *K. oxytoca*, negative for *K. pneumoniae*), gas production from lactose at 44.5 °C (positive for *K. pneumoniae*, negative for *K. oxytoca*), growth at 10 °C (positive for *K. oxytoca*, negative for *K. pneumoniae*) [19]. The degradation of pectate [20] was used to analyze collection strains along with type ones. The cultures were spotted on pectate-containing semisolid PEC-SSA medium, and pectolytic ones exhibited indenting the agar.

PCR and primer design. In this study, the tests were carried out by using lysed cells of approximately $1.0 \cdot 10^{7-8}$ cfu/ml in PCR mixtures with annealing temperature of 58 °C and $MgCl_2$ concentration of 2.5 mM. The primer sequences PEH-A (5′-ggactacgccgtctatcgtcaag-3′), PEH-B (5′-aatatccagggtcatatcgctgtg-3′), PEH-C (5′-gatacggagtatgcctttacggtg-3′), PEH-D (5′-tagcctttatcaagcggatactgg-3′) were chosen in the coding part of the gene. The primer and available

Table 1
Bacterial strains used in experiments Bacterial Strains

Bacterial Strains	Source
K. oxytoca VN13	Laboratory collection
K. oxytoca ATCC 13183	ATCC
K. planticola ATCC 33531	ATCC
K. pneumoniae ATCC 13183	ATCC
K. pneumoniae CDC 50231	Institute for Genetics of Bayreuth University (FRG)
K. pneumoniae M5a1	Institute for Microbiology of Bayreuth University (FRG)
E. carotovora subsp. *carotovora* 8982	Institute of Microbiology and Virology (IMV) of NASU (Kyiv)
E. carotovora subsp. *atroceptica* 9027	IMV NASU (Kyiv)
E. chrysanthemi 8183	IMV NASU (Kyiv)
E. coli JM109	Laboratory collection
Klebsiella sp. 030039, 30041, 030042, 030044	Kyiv Research Institute of Epidemiology and Infection Diseases
K. pneumoniae NCTC* 5054 (K1), NCTC 5055 (K2), NCTC 9128 (K8), NCTC 9130 (K10), NCTC 9131 (K11), NCTC 9132 (K12), NCTC 9136 (K16), NCTC 9138 (K18), NCTC 9141 (K21), NCTC 9148 (K28), NCTC 9150 (K30), NCTC 9157 (K37), NCTC 9161 (K41), NCTC 9175 (K55), NCTC 9186 (K66), PZH** (K69), PZH (K75), PZH (K76), PZH (K79)	Kyiv Research Institute of Epidemiology and Infection Diseases

*National Collection of Typical Cultures (London, UK); **Panstwowi Zaklad Hygieny (Institute of Hygiene, Warsaw, Poland).

sequences were compared according to the Blast N program of the Blast service at NCBI and FASTA one of the EMBO network services. Priming oligonucleotides were synthesised at the Institute of Bioorganic Chemistry of RAN (Russian Federation).

Results and Discussion Earlier the *pehX* gene of *K. oxytoca* VN13 has been cloned and sequenced (accession No AY06648) [21]. The sequence was compared with the other *peh* gene sequences available in GenBank, and the comparison did not display essential homology in the DNA sequences of pectinolytic bacteria. However, alignment of the encoded amino acids revealed 52—71 % homology with the *Yersinia enterocolitica*, *E. chrysanthemi*, and *Ralstonia solanacearum* exopolygalacturonases.

PCR-mediated method for detection of K. oxytoca. Our approach utilizes the PCR-mediated technique that allows determination of a specific gene in a living organism or a sample. The genetic marker

pehX having been identified, permits the specific detection of *K. oxytoca* in any sample and discrimination of the bacterium from related strains. The set of strains, such as type strains of the genus *Klebsiella* (*K. oxytoca* ATCC 13183, *K. planticola* ATCC 33531 and *K. pneumoniae* ATCC 13883), well characterized strains *K. pneumoniae* M5a1 and *K. oxytoca* VN13 [1, 6], as well as strains of the genus *Erwinia*, and *E. coli* JM109 analyzed previously by the method of pectate degradation, were used in experiments to verify specificity of the primers designed. All strains of *Erwinia* sank into the medium, as well as the *K. oxytoca* strains and *K. pneumoniae* M5a1 (Fig. 1). Other strains of *Klebsiella* grew but did not indent the medium. The *K. oxytoca* VN13 and *K. oxytoca* ATCC 13183 strains produced the 344 and 513 bp amplicons in PCR with a pair of primers, PEH-C, D, and PEH-A, D (Fig. 2, *A*, *B*). The *K. pneumoniae* ATCC 13883, *E. carotovora* subsp. *carotovora*, *E. carotovora* subsp. *atroceptica*, *E. chrysanthemi*, *K. planticola* ATCC 33531 and *E. coli* JM109 were negative in the PCR tests. The nitrogen-fixing strain of *K. pneumoniae* M5a1 possessing characteristics of *K. oxytoca*, also generated specific amplicons of 344 and 513 bp (Fig. 2, *C*). DNA of diazotrophic bacterium *K. pneumoniae* 342, correctly identified by molecular methods [8], was used as a reference in experiments to detect the *pehX* specific sequence in bacterial DNA. The primers designed for the *pehX* gene did not anneal to DNAs of *K. pneumoniae* 342, *K. pneumoniae* ZMVSY and the clinical isolate *K. pneumoniae* MGH78578.

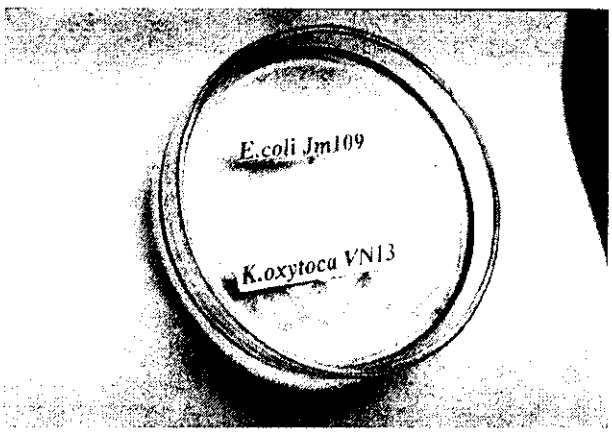

Fig. 1. Capability of *Klebsiella oxytoca* to indent a polygalacturonate semisolid agar as evidence for pectinase production

Amplification of the unique DNA fragments in the PCR test revealed high level of specificity of the primers designed for the *pehX* gene. The negative results in *E. carotovora* and *E. chrysanthemi* strains were expected because there was practically no homology between the polygalacturonase-encoding gene of *K. oxytoca* and analogous genes of *Erwinia* [21]. The absence of amplicons in *K. pneumoniae*, *K. planticola* and *E. coli* JM109 can be explained by the absence of specific gene encoding the hydrolytic enzyme.

The method presented can be used for the detection of specific target bacteria in a complex sample mixture. The latter may contain a variety of components including non-target or background microorganisms. The method proceeds by first culturing the complex sample mixture in a non-selective growth medium followed by the PCR-mediated detection of a target bacterial DNA. The target DNA is detected via a DNA amplification protocol with a primer pair selected to amplify a specific portion of the target bacterial DNA. A control DNA is amplified in parallel with the target bacterial DNA. The control DNA is

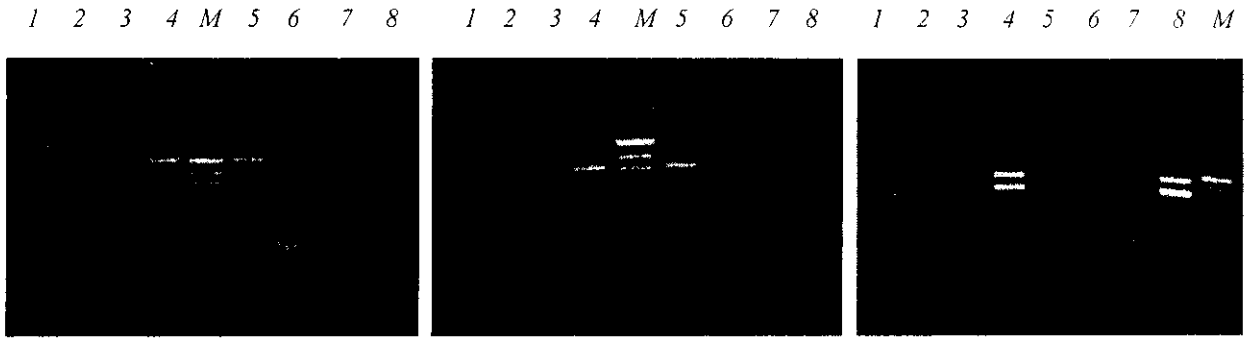

Fig. 2. Specific 513-bp (*A*), 344-bp (*B*), 451 and 344-bp (*C*) the *peh* gene fragments generated with PCR_s (*A*, *B*: *1* — *E. coli* JM109; *2* — *K. pneumoniae* ATCC 13883; *3* — *K. planticola* ATCC 33531; *4*- — *K. oxytoca* VN13; *M* — *pUC19/HpaII* (marker); *5* — *K. oxytoca* ATCC 13183; *6* — *E. carotovora* subsp. *carotovora* 8982; *7* — *E. carotovora* subsp. *atroceptica* 9027; *8* — *E. chrysanthemi* 8183; C: *1* — *K. pneumoniae* PZH (K79); *2* — *K. pneumoniae* NCTC 9130 (K10); *3* — *K. pneumoniae* NCTC 9138 (K18); *4* — *K. pneumoniae* M5a1; *5* — *K. pneumoniae* ZMVSY; *6* — *K. pneumoniae* MGH78578; *7* — *K. pneumoniae* 342; *8* — *K. pneumoniae* NCTC 9186 (K66); *M* — *pUC19/HpaII* (marker))

Table 2
Differentiation of Klebsiella spp. by biochemical tests

Strain	Trait					
	Growth at 10 °C	D-Melezitose	Gas production from lactose at 44.5 °C	Indole production	Pectate degradation	Presence of a *pehX* sequence
K41	+	+	—	+	+	+
K66	+	+	—	+	+	+
K69	+	—	—	+	+	+
K75	+	+	—	+	+	+
K76	+	+	—	+	+	+
K79	+	+	+	+	+	+
K10	—	—	+	—	—	—
K21	—	—	+	—	—	—
K28	—	—	+	—	—	—
K30	—	—	+	—	—	—
K37	—	—	+	—	—	—
K55	—	—	+	—	—	—
K. pneumoniae ATCC 13883	—	—	+	—	—	—
K. oxytoca ATCC 13183	+	+	—	+	+	+
K. oxytoca VN13	+	—	—	+	+	+

specifically designed to be amplified with the primers that are identical to the primers used in the amplification of the target genomic DNA. Use of this control validates the amplification reaction. Detection of the amplified target DNA and the control is accomplished by gel electrophoresis.

Screening collection of Klebsiella by a molecular method. A set of bacterial strains of the genus *Klebsiella*, deposited in the collection of Kyiv Research Institute of Epidemiology and Infection Diseases, both type strains gathered from international collections and clinical isolates have been screened for the availability of the *pehX* gene. Five strains, deposited as *K. pneumoniae* (type strains K41, K66, K69, K75, K76), generated specific *K. oxytoca* amplicons with the primers PEH-A, B and PEH-C, D (see Fig. 2, *C*). DNA of *K. pneumoniae* PZH (K79) did give a 344 bp fragment, but did not give a 451 bp one which may be explained either by some deletion in the gene, or by a lack of homology between strains VN13 and PZH (K79) within sites of the primers PEH-A and PEH-B recognition.

Identification of K. oxytoca or K. pneumoniae by biochemical tests. Six *Peh*$^+$ strains were examined by the biochemical key tests listed above that differentiate *K. oxytoca* from *K. pneumoniae*. In parallel six type strains of *K. pneumoniae* (K10, K21, K28, K30, K37, K55) that did not possess the *pehX*-specific sequence in their genomes were included in biochemical testing. The results of these tests correlated with the data of the *peh* DNA amplification and confirmed the taxonomic positions of six strains previously identified by molecular methods as *K. oxytoca* and six strains identified as *K. pneumoniae* (Table 2). This means that six type strains of *K. pneumoniae* deposited in international collections were identified earlier according to an outdated taxonomic system.

Conclusions. The rapid and sensitive PCR test for the discrimination of *K. oxytoca* between closely related bacteria and detection of this bacterium in environmental and clinical samples has been elaborated. The test is based on the recognition of unique DNA sequence within the gene encoding the polygalacturonase that cleaves a polygalacturonic chain of demethoxylated pectin. The use of different pairs of primers specific to the *pehX* gene helps to avoid mistakes in differentiation of *K. oxytoca*. The diagnostic test gives a rapid answer, regarding the presence of *K. oxytoca* in a sample, or in differentiating this bacterium from some other bacteria, such as *K. pneumoniae*, with which they can be confused. The specific PCR test overcomes the handicap of time-consuming microbiological and biochemical methods

and can be used for ecological monitoring *K. oxytoca* and in medical laboratories.

Acknowledgements. We are grateful to Eric Triplett (Wisconsin University) for providing the *K. pneumoniae* DNA and to Mike Merrik (John Innes Centre) for critical reading of the manuscript.

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ПЛР-опосередкований метод визначення *Klebsiella oxytoca* серед близьких бактерій у природних та медичних зразках

Резюме

На основі ПЛР-ампліфікації унікальних послідовностей ДНК гена, що кодує ферменті полігалактуроназу (pehX), розроблено специфічний метод для вирізнення бактерії K. oxytoca серед інших бактерій роду Klebsiella. Чотири пари праймерів створено для отримання ампліконів 282, 344, 451 та 513 п. н. Специфічність тесту підтверджено відсутністю продуктів ПЛР у близьких бактерій K. pneumoniae, K. planticola та видів роду Erwinia, що розкладають полігалактуронат. ПЛР-тест дозволяє швидко визначити наявність K. oxytoca у зразку або відрізнити цю бактерію від представників інших видів, наприклад, від K. pneumoniae, яка дуже схожа на неї. Діагностичний тест може бути використано в екологічному моніторингу K. oxytoca, а також у медичних лабораторіях.

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ПЦР-опосредованный метод для определения *Klebsiella oxytoca* среди близких бактерий в природных и медицинских образцах

Резюме

На основе ПЦР-амплификации уникальных последовательностей ДНК гена, кодирующего фермент полигалактуроназу (pehX), разработан метод для выявления бактерии K. oxytoca среди других бактерий рода Klebsiella. Четыре пары праймеров созданы для получения ампликонов размером 282, 344, 451 и 513 пар нуклеотидов. Специфичность теста подтверждена отсутствием продуктов ПЦР у близких бактерий K. pneumoniae, K. planticola и видов рода Erwinia, разлагающих полигалактуронат. ПЦР-тест позволяет быстро определить наличие K. oxytoca в образцах или отличить эту бактерию от представителей других видов, например, от K. pneumoniae, которая очень на нее похожа. Диагностический тест может быть использован в экологическом мониторинге K. oxytoca, а также в медицинских лабораториях.

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УДК 577.21
Надійшла до редакції 01.03.02