Curr Microbiol (2016) 72:570–582
DOI 10.1007/s00284-016-0993-0

Application of Routine Diagnostic Procedure, VITEK 2 Compact, MALDI-TOF MS, and PCR Assays in Identification Procedure of Bacterial Strain with Ambiguous Phenotype

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Received: 3 September 2015 / Accepted: 19 December 2015 / Published online: 25 January 2016

Abstract In diagnostic microbiology as well as in microbiological research, the identification of a microorganism is a crucial and decisive stage. A broad choice of methods is available, based on both phenotypic and molecular properties of microbes. The aim of this study was to compare the application of phenotypic and molecular tools in bacterial identification on the example of Gram-negative intestine rod with an ambiguous phenotype. Different methods of identification procedure, which based on various properties of bacteria, were applied, e.g., microscopic observation of single-bacterial cells, macroscopic observation of bacterial colonies morphology, the automated system of microorganism identification (biochemical tests), the mass spectrometry method (analysis of bacterial proteome), and genetic analysis with PCR reactions. The obtained results revealed discrepancies in the identification of the tested bacterial strain with an atypical phenotype: mucous morphology of colonies, not characteristic for either *E. coli* and *Citrobacter spp.,* mass spectrometry analysis of proteome initially assigned the tested strain to *Citrobacter genus* (*C. freundii*) and biochemical profiles pointed to *Escherichia coli.* A decisive method in the current study was genetic analysis with PCR reactions which identified conserved genetic sequences highly specific to *E. coli* species in the genome of the tested strain.

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Introduction

Conventional identification procedure of *Enterobacteriaceae* family bacteria to genus or species level, commonly used in diagnostic laboratories, relies on macroscopic characteristic of bacterial growth on selective media. This traditional culture-dependent procedure bases on unique or distinctive biochemical properties of enteric bacteria such as fermentation of lactose, abilities to utilize citrate as a carbon source, production of hydrogen sulfide (H_2S), degradation of urea, and abilities to convert tryptophan into indole [20, 24]. However, identification of bacteria, even the well-studied ones such as *Enterobacteriaceae* rods, only by description of colony morphology is no longer used as a reliable method. Automation of biochemical tests has shortened the identification time to 2–10 h (3 h for Gram-negative rods), improved reliability and increased efficiency, with minimal manual preparation of samples in comparison to manual miniaturized biochemical tests [15]. VITEK 2 Compact is one of the most widely used integrated and automated systems in bacterial identification based on biochemical profiles of tested strains. This fluorescence and/or colorimetry-based system in combination with compact plastic cards containing small quantities of selective or differentiated media or reagents enables bacterial identification in shorter time than with conventional methods [3].

Molecular analysis of microorganisms has progressed far beyond the level needed for most routine diagnostic

laboratories. This method facilitates not only determination of bacterial species but also differentiation of strains of the same species—typing and precise analysis of their phylogeny. Molecular approaches to rapid identification of target microorganism basing on genetic diversity include a wide range of methods [12]. The application of polymerase chain reaction (PCR) has revolutionized the bacterial identification procedure. PCR is one of the most sensitive and commonly used methods allowing rapid and highly sensitive detection of microorganisms, even those that are either difficult to identify with conventional bacteriological methods or viable but non-culturable under in vitro conditions. PCR-based identifications rely on amplification of conserved genes, specific to certain microorganisms such as those encoding elongation factors, RNA polymerase (*rpoB*) or ribosomal DNA genes, followed by detection of species-specific sequences in the products of amplification. As the example of *Escherichia coli* shows, bacteria belonging to this species can be identified by detection of conserved genetic sequences such as *16 S rRNA, 16 S rRNA ITS 23 S rRNA*, or *uidA* gene, which were experimentally proved as specific to *E. coli* species [12, 27].

Next to genetics, the second pillar of molecular techniques of bacteria identification is proteomics. One of the most innovative and revolutionary molecular analytic tools used in bacteriological diagnostic is Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS). The MALDI-TOF MS identification relies on the analysis of bacterial proteins, mainly ribosomal proteins, and peptides that are highly conserved and abundant in the certain bacteria cell [10]. In comparison to conventional identification methods, MALDI-TOF MS has the advantage of identifying microorganisms directly from colonies grown on culture plates within minutes, with simple procedures, low-sample volume requirements, and no sophisticated or expensive reagents. This advanced method is characterized by significant benefit—considerably shorter microorganism identification time, high accuracy, and minimized influence of environmental conditions or microorganism growth on the result.

However, MALDI-TOF MS has limitations in connection with low differentiation of closely related microbial isolates, e.g., identification of microorganisms in mixed-culture samples, or limitations in connection with a lack of appropriate mass spectra in the database. In MALDI-TOF MS analysis, there are "protein fingerprint" signatures generated from whole bacterial cells. These fingerprints are compared to a database of reference spectra by means of various algorithms [18]. MALDI-TOF MS has been used to identify a broad range of different bacterial genera among Gram-negative rods, such as *E. coli* and other bacteria of the *Enterobacteriaceae* family as well as Gram-positive bacteria such as *Staphylococcus aureus, Streptococcus spp., Bacillus cereus*, or *Listeria spp*. It has also been demonstrated that MALDI-TOF mass spectrometry is effective in discriminating ampicillin-resistant *E. coli* organisms displaying a peak assigned to β-lactamase [6, 25].

Escherichia coli is not a single clone and the population of *E. coli* includes commensal strains of intestinal microflora of warm-blooded animals, including humans, as well as highly specialized or opportunistic pathogens able to develop a wide range of infections (from diarrheal diseases to extraintestinal infections) [4, 31]. *E. coli* species is an example showing that interactions between bacteria and host are very flexible, e.g., *E. coli* are able to fluctuate between commensalism and pathogenesis by horizontal transfer of genes [35]. Organisms of warm-blooded animals are a typical niche for *E. coli,* even for its commensal strain as well as pathogenic clones. However, there is still insufficient knowledge about the epidemiology of *E coli* strains among cold-blooded animals such as reptiles [2, 37].

The aim of this study was a comparison of different both phenotypic and molecular methods that are used for the identification of bacteria on the example of Gram-negative rod with an ambiguous phenotype. For this purpose, we applied a set of identification methods based on different properties of bacteria such as colony morphology (macroscopic observation of bacterial growth on culture media), metabolism activity and biochemical properties (automated system of microorganisms identification VITEK Compact 2), analysis of bacterial proteome (MALDI-TOF MS Biotyper) and genetic analysis of bacteria (species-specific PCR reactions). The results of preliminary identification procedure of tested strain with the use of conventional bacterial identification with culture media and MALDI-TOF MS were discrepant, and pointed to *E. coli* and *Citrobacer spp.* respectively. Because of that, we chose strains of *E. coli* and *Citrobacter spp*. as control strains in further analysis. The presented research indicates that a combination of methods based on different technologies should be applied for the identification and characterization of microbial isolates with an atypical phenotype.

Materials and Methods

Bacterial Strain

Tested Bacterial Strain

The strain indexed as 305C (the Collection of the Department of Microbiology, Institute of Genetic and Microbiology, University of Wrocław) displaying an ambiguous phenotype was isolated from feces of a healthy *Kinixys belliana* (bell's hinge-back tortoise), an African tortoise, belongs to the family *Testudinidae* dwelled in Zoological Garden in

Wrocław and came from exchange with other ZOO. The strain of *E. coli* 305C is also deposited at Polish Collection of Microorganisms with number PCM 2792.

Control Bacterial Strains

Control strains of *E. coli* species used in presented study:

A reference strain of *E. coli* PCM 172 (the Polish Collection of Microorganisms).

A strain of *E. coli* indexed as 209E isolated from feces of a healthy *Lamprophis fuliginosus* an African house snakes, dwelled in Zoological Garden in Wrocław. The strain of *E. coli* 209E is also deposited at Polish Collection of Microorganisms with number PCM 2793.

Control of *Citrobacter spp.* used in presented study:

A reference strain of *Citrobacter spp.—Citrobacter freundii* PCM 1527.

A strain of *Citrobacter spp.* indexed as 303C isolated from feces of a healthy *Coelognathus radiates,* a radiated ratsnake dwelled in ZOO in Wroclaw. The strain of *Citrobacter braakii* 303C is also deposited at Polish Collection of Microorganisms with number PCM 2794.

All examined strains: bacterial isolate with ambiguous phenotype 305C, *E. coli* PCM 172, *Citrobacter spp* PCM 1527, *E. coli* 209E, and *Citrobacter spp.* 303C has been banked and stored in glycerin-broth in temperature of −70 °C in Microbiology Department of Institute Genetic and Microbiology and recovered from storage by overnight incubation in Brain–Heart Infusion (BHI) medium (Biocorp) in 37 °C with shaking.

Identification of Presumptive *E. coli* Strains by Conventional Methods

The tested strain 305C and control strains were characterized both at a phenotypic level by the description of colony morphology, Gram-staining and the positive–negative staining method. Bacterial strains after overnight incubation in aerobic conditions in Brain–Heart Infusion (BHI) broth (Biocorp) in 37 °C with shaking were inoculated on selective media as ENDO Agar (Biocorp), MacConkey Agar (Biocorp), Simmons agar (Biocorp). After overnight growth, usually for 18–24 h incubated at 37 °C the morphology of colonies and growth characteristic on agar plates were evaluated. There were also performed a Gram-staining and positive–negative staining of examined strains.

Positive–Negative Staining (Capsule Staining)

Pure cultures of tested bacterial isolate and control strains after overnight incubation in 37 °C on agar plates were inoculated onto a clean slide in sterile saline. First bacterial suspension were stained with safranin for 25 min. Next, one drop of nigrosin was added and mixed, and the suspension was spread the drop out with another clean slide. Dried slide were observed using optical microscope [MN800 EPI/DIA], including oil immersion. Bacterial cell were positively red stained with safranin, the background was negatively black-stained with nigrosin, and the bacterial capsules could be visible as colorless ring around the red cell on the dark background.

The Automated Systems for Identification of Microorganisms

Bacterial isolates were inoculated into the appropriate VITEK identification strip using the VITEK®2Compact (bioMérieux). Briefly, strains were cultured on MacConkey agar (Biocorp) for 18–24 h at 37 °C before were subjected to analysis. A bacterial suspension was adjusted to a McFarland standard of 0.50 in a solution of 0.45 % sodium chloride using DensiLameter, type: Densi-2, no 227/06. The time between preparation of the solution and filling of the card was always less than 1 h. Analysis was done using the identification card for Gram-negative bacteria. Cards are automatically read in every 15 min. Data were analyzed using the VITEK 2 software version VT2-R03.1. according to the manufacturer's instructions [5].

Identification with Mass Spectrometry Methods

Sample Preparation for MALDI-TOF MS Analysis

Two to five single colonies of each isolate were taken directly from nutrient agar (Biocorp) plate after overnight (for 18–24 h) incubation for preparation of bacterial sample. Bacterial were suspended in 300 µl of double-distilled water, and 900 µl of absolute ethanol was added. The samples were centrifuged (13,000 g, 5 min), and the supernatant was completely removed under vacuum. Lysates were prepared by adding 50 µl 70 % formic acid to the bacterial pellet and mixing thoroughly, before adding 50 µl acetonitrile and again mixing thoroughly. Following centrifugation (13,000 g, 2 min), the supernatant was transferred to a fresh tube, and then 1 µl of the supernatant containing the bacterial lysate was transferred to a sample position on a 384 ground steel MALDI target plate (BrukerDaltonics) and air-dried at room temperature. The sample was overlaid with 1 µl of MALDI matrix solution: a saturated solution of α-cyano-4-hydroxy-cinnamic acid (HCCA, BrukerDaltonics) in 50 % acetonitrile with 2.5 % TCA (trifluoroacetic acid).

MALDI-TOF MS Analysis Procedure

Identification of bacterial strain using MALDI–TOF MS Biotyper were conducted with application of two models of

MALDI-TOF: AUTOFLEX III SmartBean (BrukerDaltonics) and UltraflExtreme (BrukerDaltonics). The appliances vary in type of laser source, version of analyzing software and the version of MALDI-Biotyper library database, as following:

- AUTOFLEX III SmartBean serial number #245584 vertical MALDI-TOF MS (BrukerDaltonics) mass spectrometer with fuzzy control of Ion source 20 kV; a 337-nm nitrogen laser with a frequency: 100.0 Hz; detector gain, 1780 V; and gating, maximum, 1500 Da.
- UltraflExtreme serial number # 259901 (BrukerDaltonics) mass spectrometer with fuzzy control of Ion source 20 kV; equipped with the innovative smartbeam-II™ laser with frequency of 1000.0–2000.0 Hz; detector gain, 1780 V; and gating, maximum, 1500 Da.

For both models, spectra were recorded in the positive linear mode for a mass range of 2000–20,000 Da. Each spectrum was obtained by averaging 600 laser shots acquired from the automatic mode under control of FlexControl software ver. 3.3 and 3.4 (BrukerDaltonics), respectively. The spectra were externally calibrated using an *E. coli* DH5-alpha standard (BrukerDaltonics). The calibrant consisted of seven ribosomal proteins from *E. coli* with added RNAse A and myoglobin to cover a range of 3637.8–16957.4 Da. Biotyper ver. 3.0 (MSP 4613) and ver. 3.1 (MSP 4613) database software (BrukerDaltonics), respectively, were used for the identification of bacterial isolates.

The identification of the tested strain with the application of both mass spectrometer appliance models was conducted (with UltraflExtreme repeated threefold) with the same procedure, the same growth conditions of bacterial strain and extraction method.

Genetic Analysis

DNA Extraction

Total bacterial DNA from overnight (for 18–24 h) culture in nutrient broth, for PCR assays, was extracted using commercially available Genomic Mini kit (A&A Biotechnology), according to the extraction protocol provided by the manufacturer with minor own modifications.

Single Primer PCR Specific for Species of E. coli

The set of PCR reactions were conducted according to literature [28] with primers recognizing genetic sequences specific to *E. coli* species such as a gene *uidA*—encoding the β-glucuronidase; the genetic region of conserved gene of 16SrRNA; and the internal region of 16S-ITS-23S between 16SrRNA and 23SrRNA genes, which were obtained from Genomed. Primers, their position within the target sequences, the size of amplicons, and the biological role of targeted genes are presented in Table 1.

For each reaction, there was separately setup 25-µl PCR reaction mixture per sample containing 50 ng of template DNA, 1 U of Taq polymerase (Thermo Scientific), 2.5 µl of 10× Thermo DNA buffer (Thermo Scientific), 200 mM dNTP (Thermo Scientific), and 20 pmol each of the specific—*E. coli* species primers (Genomed). The reactions mixtures were subjected separately to the following cycling conditions: 95 °C for 3 min and 30 cycles of: denaturation (30 s, 95 °C), annealing (30 s, 62 °C for *uidA*; 60 °C for 16S rRNA region; and 57 °C for 16S-ITS-23S region, respectively), extension steps (30 s, 72 °C), and final extension (5 min, 72 °C).

Phylogenetic Typing

To assess genetic diversity, phylogenetic groups of tested *E. coli* strains were determined by triplex-PCR method according to Clermont [8], with primers targeting genes: *chuA*, *yjaA* and a genetic region with unknown function: *TspE4C2* obtained from Genomed. Primers, their position within the target sequences, the size of amplicons, and the biological role of targeted genes are presented in Table 1. For the reaction, there was set up 25-µl PCR mixture per sample containing 50 ng of template DNA, 1 U of Taq polymerase (Thermo Scientific), 2.5 µl of 10 × Thermo DNA buffer (Thermo Scientific), 200 mM dNTP (Thermo Scientific), and 20 pmol each of the primers (Genomed). The cycling conditions were as follows: initial denaturation at 95 °C for 4 min and 35 cycles of denaturation (30 s, 95 °C), annealing (30 s, 52.5 °C), extension steps (1 min, 72 °C) and final extension (5 min, 72 °C).

Virulence Genotyping

Additionally to the methods of identification of examined bacterial strains, there were subjected to testing of 14 virulence genes related to pathogenicity of *E. coli* such as *astA*, *iss*, *irp2*, *cva/cvi*, *iucD*, *papC*, *tsh*, *vat*, *fyuA*, *stx2f*, *hlyE*, *fimC*, *eae*, and *bfp* were targeted by three PCR reactions using the protocol given below according to literature [13, 21] with own modifications. Primers (Genomed), their position within the target sequences, the size of amplicons, and the biological role of targeted genes are presented in Table 1. For multiplex with primers recognizing sequences: *astA*, *iss, irp2 papC, iucD, tsh, vat*, and *cva/cvi* and for multiplex with primers recognizing sequences: *stx2, hlyE, fimC, fyuA*, the cycling conditions were the same and setup as follows: 95 °C for 4 min and 35 cycles of denaturation (30 s, 95 °C), annealing (30 s, 54 °C), extension steps (30 s, 72 °C), and final extension

Table 1 Primers used in PCR virotyping reactions with their position within detected sequence, size of amplicons, and biological function of targeted genes

Gene target	Primer sequence	Amplicon size (bp)	References	Biological role of amplified genetic sequence
uidA	5′-TGGTAATTACCGACGAAAACGGC-3′	147	[36]	The β-glucuronidase enzyme
	5′-ACGCGTGGTTACAGTCTTGCG-3′			
16SrRNA	5′-GGGAGTAAAGTTAATACCTTTGCTC-3′	583	[38]	Conserved region of 16S rRNA
	5′-TTCCCGAAGGCACATTCT-3′			
	5′-TTCCCGAAGGCACCAATC-3′			
16S-ITS-23S	5′-CAATTTTCGTGTCCCCTTCG-3′	450	[23]	The internal-transcribed spacer (ITS) region t between the 16S and 23S rRNA genes
	5′-GTTAATGATAGTGTGTCGAAAC-3			
chuA,	5′-GACGAACCAACGGTCAGGAT-3′	279	[8]	Heme transport in a bacterial cell
	5′-TGCCGCCAGTACCAAAGACA-3′			
yjaA	5′-TGAAGTGTCAGGAGACGCTG-3′	211	[8]	Unknown function
	5′-ATGGAGAATGCGTTCCTCAAC-3′			
TspE4C2	5′-GAGTAATGTCGGGGCATTCA-3′	152	[8]	Unknown function
	5′-CGCGCCAACAAAGTATTACG-3′			
astA	5′-TGCCATCAACACAGTATATCC-3′	116	[13]	Heat stable toxin
	5′-TCAGGTCGCGAGTGACGGC-3′			
iss	5′-ATCACATAGGATTCTGCCG-3′	309	[13]	Increased serum survival protein
	5′- CAGCGGAGTATAGATGCCA-3′			
irp2	5′-AAGGATTCGCTGTTACCGGAC-3′	413	[13]	Siderophore, iron uptake system
	5′- AACTCCTGATACAGGTGG-3′			
cva/cvi	5′-TGGTAGAATGTGCCAGAGCAA-3′	680	[13]	Colicin production
	5′-GAGCTGTTTGTAGCGAAGC-3′			
iucD	5′-ACAAAAAGTTCTATCGCTTCC-3′	714	[13]	Siderophore
	5′-CCTGATCCAGATGATGCTC-3′			
papC	5′-TGATATCACGCAGTCAGTAGC-3′	501	[13]	P fimbriae
	5′-CCGGCCATATTCACATAA-3′			
tsh	5′-ACTATTCTCTGCAGGAAGTC-3′	824	[13]	Temperature-sensitive hemagglutinin
	5′-CTTCCGATGTTCTGAACGT-3′		[13]	
vat	5′-TCCTGGGACATAATGGTCAG-3′	981	[13]	Transporter of cytotoxin
	5′-GTGTCAGAACGGAATTG-3′			
stx2f	5′-ATC CTA TTC CCG GGA GTT TAC G-3′	496	[33]	Shiga toxin
	5′-GCG TCA TCG TAT ACA CAG GAG C-3′			
fyuA,	5′- GGCGGCGTGCGCTTCTCGCA-3′	776	[19]	Siderophore
	5′-CGCAGTAGGCACGATGTTGTA-3′			
hlyE	5′-GGTGCAGCAGAAAAAGTTGTAG-3′	1551	[19]	α-hemolysin
	5′-TCTCGCCTGATAGTGTTTGGTA-3′			
fimC	5′-GTTGATCAAACCGTTCAG-3′	331	[19]	Fimbriae type 1
	5′-AATAACGCGCCTGGAACG-3′			
eae	5′-TCA ATG CAG TTC CGT TAT CAG TT-3′	482	[40]	Intimine
	5′-GTA AAG TCC GTT ACC CCA ACC TG-3′			
bfp	5′-GGA AGT CAA ATT CAT GGG GGT AT-3′	300	[40]	Bundle-forming pili
	5′-GGA ATC AGA CGC AGA CTG GTA GT-3′			

(10 min, 72 °C). For multiplex with primers recognizing sequences: *eae* and *bfp*, the cycling conditions were as follows at 95 °C for 3 min and 35 cycles of denaturation (30 s, 95 °C), annealing (1:30 min, 55 °C), extension steps (1 min, 72 °C), and final extension (5 min, 72 °C).

Gel Electrophoresis, Visualization, and Analysis of PCR Amplification Products

PCR amplifications of each type of reaction were performed with a DNA Thermal Cycler T100tm (Bio-Rad).

The amplified products from all types of the PCR-based typing methods and samples containing known amounts of marker DNA (100 bp ladder; Sigma-Aldrich) were resolved on a 2 % agarose gel (Sigma-Aldrich) and visualized by staining with Midori Green DNA (Nippon Genetics) stain using a Gel-Doc UV transilluminator system (Bio-Rad), and analyzed with Quantity One software (Bio-Rad). For each PCR- reaction, as a negative control, there was used 1 μL of sterile water added to the PCR mixture. PCR assays were performed twice to ensure that the strains were correctly assigned to their respective patterns.

Results

Description of Colonies Morphology and Growth Characteristic on Diagnostic Media

Strain 305C on nutrient agar and on MacConkey agar produced milky-white and rose-pink colonies, respectively, with viscous and highly mucous appearance as shown in Fig. 1a, b. This mucous morphology of colonies is considered as not characteristic either of *E. coli* and *Citrobacter spp.* In contrast, both *E. coli* PCM 172 and 209E *E. coli* grew as colorless or slightly white and rose-pink-round colonies on nutrient agar and on MacCokey agar, respectively (Fig. 1c); *Citrobacter spp.* 303C and as well as *Citobacter spp.* PCM 1527 strains grew both as colonies strongly similar to typical *E. coli* colonies (Fig. 1d). Strain 305C, as well as 209E and *E. coli* PCM 172, gave colonies with greenish metallic sheen on the ENDO agar, rose-pink colonies on the MacConkey agar and were lactose fermenting and citrate negative on Simmons agar, respectively. This set of properties enables initial recognition of strain 305C as a strain that belongs to *E. coli* species. *Citrobacter spp.* 303C and *Citrobacter spp.* PCM 1527 strains both grew on MacConkey agar as lactose fermenting rose-pink colonies but were citrate positive on Simmons agar.

Microscopic Observation

The results of gram-staining in microscopic observation showed that the 305C strain is Gram-negative, non-sporulating, and rod-shaped bacteria. Positive–negative staining demonstrated that the 305C isolate possesses a thin capsule, around the cell's surface, as can be seen in a microscopic photograph (Fig. 2a) In contrast, positive–negative staining of both control strain of *E. coli* and *Citrobacter spp.* revealed no capsules around the surface of the cell, which is typical of these bacterial species (Fig. 2b, c, respectively).

Identification with Automated Identification System

VITEK®2Compact (bioMérieux) method based on a panel of biochemical tests assigned 305C strain to *E. coli* species with identification with confidence on an excellent level with the probability of 99 %.

Analysis of Bacterial "Protein Fingerprints"

Results of mass spectrometry analysis obtained with two appliances of MALDI–TOF MS: AUTOFLEX III SmartBean (BrukerDaltonics) and UltraflExtreme (BrukerDaltonics) that are differed in the identification of the 305C bacterial isolate and gave slightly different score value for the control strains.

According to the first assignation with the AUTOFLEX III SmartBean (BrukerDaltonics) instrument, the 305C isolate with the highest score value of 2.271 was identified as *Citrobacter freundii,* with a species secure identification score of 2.244 as *Citrobacter braakii,* and with a secure genus identification score of 2.044 as *E. coli.* This result was verified by threefold MALDI-TOF MS analysis with UltraflExtreme (BrukerDaltonics) MALDI-TOF MS Biotyper device and with this appliance the 305C strain with the highest score value of 2.442 was identified as *E. coli.*

Mass spectrometry (MS) identification obtained with Autoflex III and with UltraflExtreme for 303C pointed to *Citrobacter* genus (*C. braakii*) with the score value of 2.193 and 2.389, respectively. Analysis of 209E strain using both MALDI-TOF MS devices identified this strain as *E. coli* with score values of 2.440 and 2.427, respectively.

Identification of Bacterial Strains by Means of Genetic Methods

The results of *E. coli* species-specific PCR assays revealed that strain 305C was positive for all three genetic sequences conserved and highly specific to *E. coli*, i.e., uidA, regions of 16SrRNA, and 16S-ITS-23S (Fig. 3a, lane 1, b, lane 1 and c, lane 1, respectively). Both *E. coli* 209E strain and *E. coli* PCM 172 strain were positive for uidA (Fig. 3a, lanes 3 and 5, respectively), 16SrRNA (Fig. 3b, lanes 3 and 5, respectively), and 16S-ITS-23S (Fig. 3c, lanes 3 and 5, respectively), while the genome of *Citrobacter spp.* 303C strain and of *Citrobacter spp.* PCM 1527 reference strain lacked these three genes or had nonspecific products of amplification (Fig. 3a, lanes 2 and 4; b, lanes 2 and 4; c, lanes 2 and 4, respectively).

Consistent with the results of triplex-PCR according to Clermont [8] based on amplicons conformation as shown in Fig. 4b, strain 305C was assigned to phylogroup A of *E. coli* (Fig. 4a, lane 1). The 209E strain of *E. coli* and *E. coli* PCM 172 strain were both assigned to phylogroup B2 (Fig. 4a,

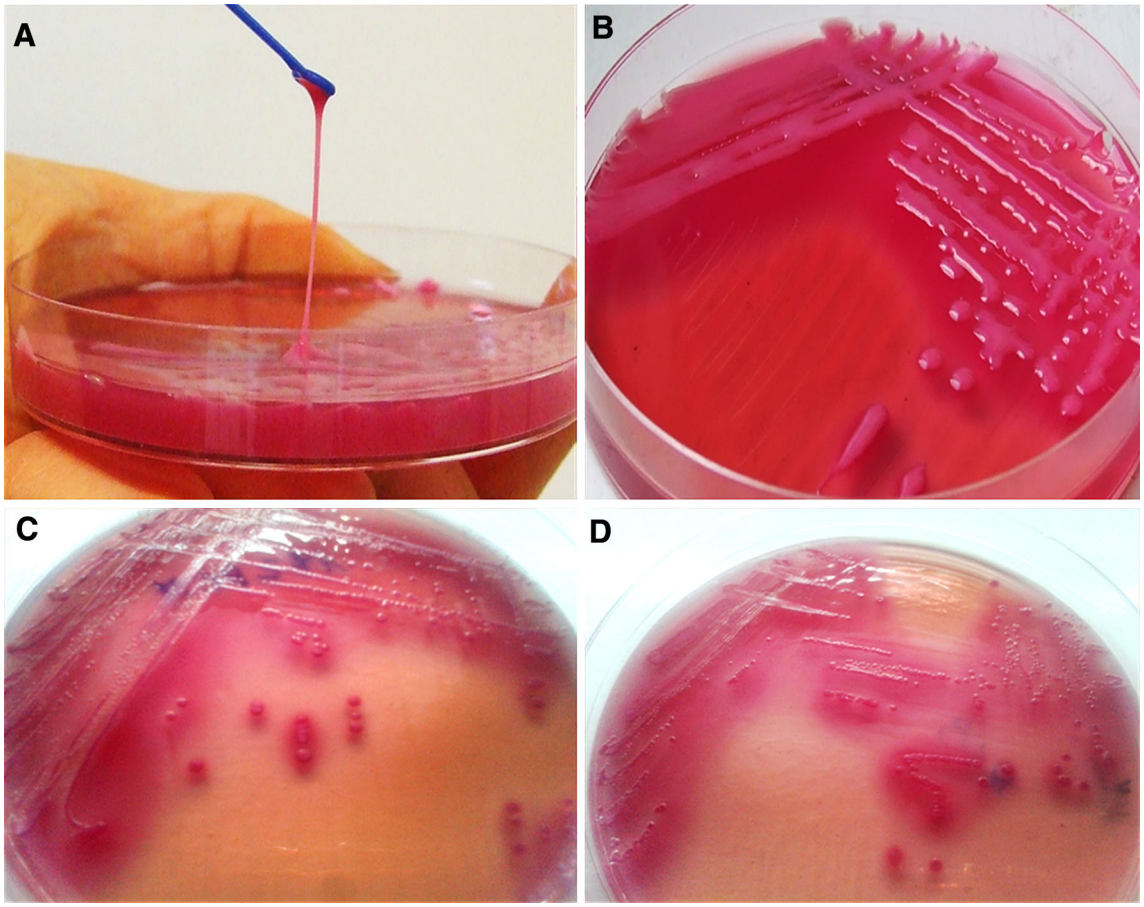


Fig. 1 Photographs of macroscopic morphology of colonies growth of tested bacterial strains, after overnight incubation at 37 °C on MacConkey agar plates of following bacterial strains: **a**, **b** *Rose-pink colonies* with mucoid surface of 305C bacterial strain with ambiguous phenotype; **c** Smooth and *rose-pink colonies* with matt surface of *Escherichia coli* PCM 172 reference strain; **d** Smooth and *rose-pink colonies* with matt surface of *Citrobacter spp.* PCM 1527 reference strain. MacConkey agar is a selective and differential medium commonly used for isolation of Gram-negative enteric rods and differentiation lactose fermenting (*pink* to *red colonies*) from non-lactose bacteria (colorless colonies) (Color figure online)

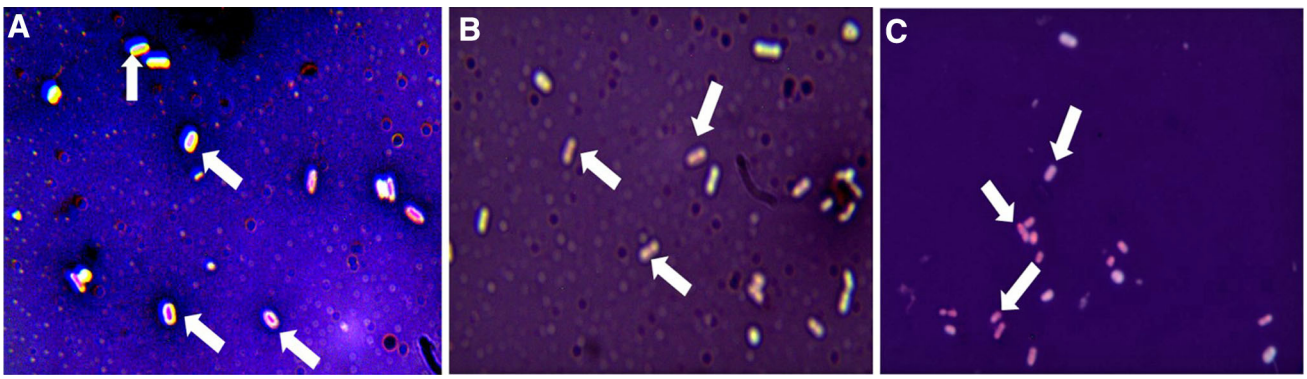


Fig. 2 Microscopic photographs [MN800 EPI/DIA] of results of positive–negative staining of the following bacterial strains: **a** 305C strain, with white arrows there are marked the bacterial cell *red* stained with safranin with light ring around them—extracellular capsules on a dark background stained with nigrosin; **b** *Escherichia coli* PCM 172 strain, with *arrows* there are marked the bacterial cells *red* stained with safranin without an extracellular capsule on a dark background stained with nigrosin; **c** *Citrobacter spp.* PCM 1527 strain, with *arrows* there are marked the bacterial cells red stained with safranin without an extracellular capsule on a dark background stained with nigrosin (Color figure online)

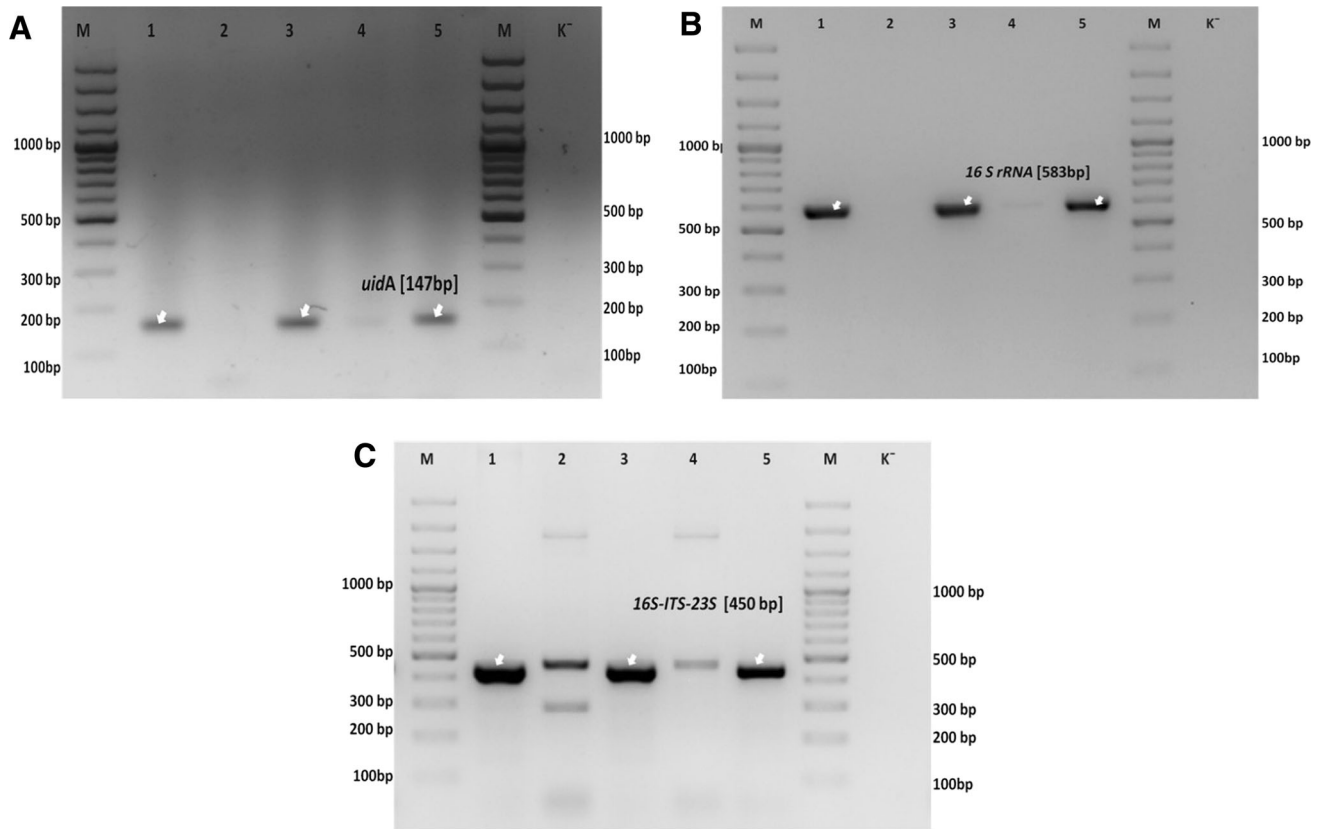


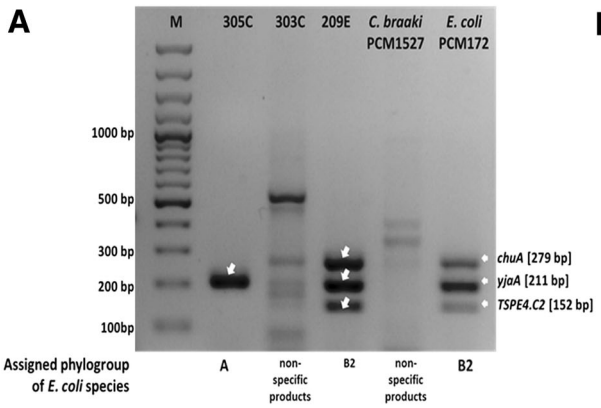
Fig. 3 Electropherograms of amplification products obtained in *E. coli* species-specific PCR reactions identifying following genetic sequences: **a** *uidA*—amplicons size ∼147 bp, gene coding the β-glucuronidase an enzyme specific for *Escherichia coli;* **b** *16S rRNA*—amplicons size ∼583 bp the conserved genetic regions of the 16S rRNA gene; **c** *16S-ITS-23S*—amplicons size ∼450 bp the internal-transcribed spacer (ITS) region between the 16SrRNA and 23SrRNA genes; In each electropherogram, there are placed and marked in the same order products of PCR obtained after amplification for following strains: *Lanes*: *1* 305C strain with ambiguous phenotype; *2* 303C *Citrobacter spp.* strain; *3* 209E *Escherichia coli* strain; *4 Citrobacter spp.* PCM 1527 reference strain; *5 Escherichia coli* PCM 172 reference strain; *Lanes* are flanked by *M*—molecular weight markers, 100 bp ladder (Sigma-Aldrich) as size standard and with K^- negative control at the end. Amplified and identified target sequences are marked with *white arrows*

lanes 3 and 5, respectively). However, nonspecific amplification products were obtained for *Citrobacter spp* 303C strain and *Citrobacter spp* PCM 1527 reference strain, which made it impossible to assign this strain to any phylogroup of *E. coli* (Fig. 4 lanes 2 and 4, respectively).

Additionally performed virulence genotyping PCR assays showed that strain 305C was characterized by virulence genes specific to *E. coli* pathogenic strains. The following products of amplification were identified for the 305C bacterial strain in PCR reactions: *irp2, fyuA, fimC*, and *eae* (Fig. 5 line 1). For the 209E strain of *E. coli*, the following genes were identified: *irp2, papC, fyuA, fimC*, and *eae* (Fig. 5 line 3). The results of PCR reactions revealed that *E. coli* PCM 172 reference strain had the following virulence genes: *irp2, papC, fimC*, and *eae* (Fig. 5 line 5), whereas 303C for *Citrobacter spp.* 303C strain and *Citrobacter spp.* PCM 1527 reference strain lacked tested virulence genes or for these strains nonspecific amplification products were obtained (Fig. 5 lines 2 and 4, respectively).

Discussion

Currently there is a broad range of tools for identifying microorganisms, which are commonly divided into phenotypic and molecular methods. Traditional techniques of microscopy, such as testing of bacteria biochemical activity or morphology observation and growth characteristic, are the basic tools in bacteriology used commonly especially for diagnostic purpose, for example, in veterinary diagnostics. However, phenotypic tools have significant limitations, as have no application in the differentiation of highly related or fastidious microbes and in an epidemiological investigations and are time and work consuming [1, 17]. Nowadays, molecular tools take priority over conventional procedures and are commonly applied because of a shorter identification time, high sensitivity, specificity, and reproducibility. Many of these methods, such as the ones based on PCR reactions, 16S ribosomal DNA sequences, hybridization, or even mass spectrometry methods and proteomics, are associated with



B

chuA	*yjaA*	*TSPE4.C2*	Phylo-group assignment
-	+	-	A
-	+	+	B1
-	-	+	B1
+	-	-	D
+	-	+	D
+	+	-	B2
+	+	+	B2

Fig. 4 Results of determination of phylogroups of *E. coli* among tested and control bacterial strains according to Clermont et al. [8] **a** Electropherogram of amplified products of triplex-PCR identifying three genes: *chuA* [279 bp], *yjaA* [211 bp], TSPE2 [152 bp]. Following *numbered lanes* contain products of triplex-PCR reaction obtained for tested and control strains. *Lanes*: *1* 305C strain with ambiguous phenotype; *2* 303C *Citrobacter spp.* strain; *3* 209E *Escherichia coli* strain; *4 Citrobacter spp.* PCM 1527 reference strain; *5 Escherichia coli* PCM 172 reference strain; *Lanes* are flanked by *M*—molecular weight markers, 100 bp ladder (Sigma-Aldrich) as size standard; and with K^- negative control at the end. **b** Table presenting the set of combinations including amplified genes allowed to the determination of phylogenetic group of a tested strains

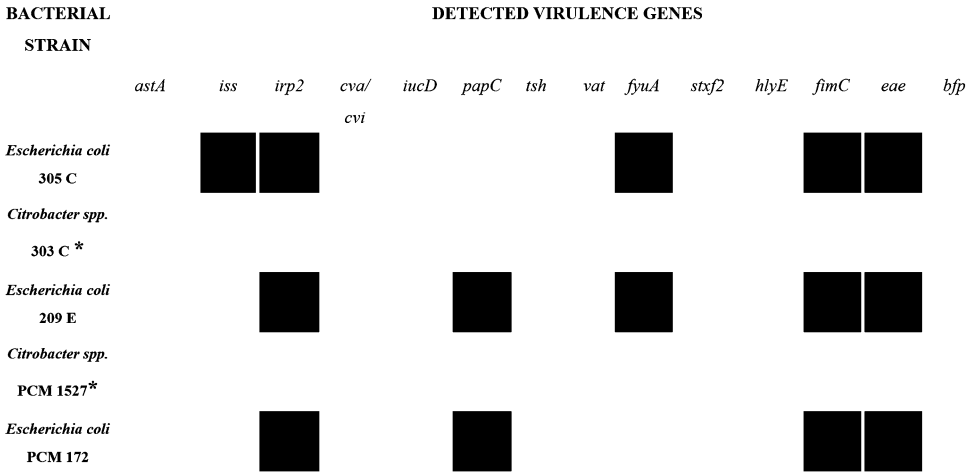


Fig. 5 Results of genetic virotyping of tested and control bacterial strains based on detecting virulence genes (VGs) characteristic for pathogenic *E. coli* strains with adequate PCR reactions. Diagram presented results of three multiplex PCR reactions amplifying following VGs. *astA* [116 bp]; i*ss* [309 bp]; *irp2* [413 bp]; *cva/cvi* [680 bp]; *iucD* [714 bp]; *papC* [501 bp]; *tsh* [824 bp]; *vat* [981 bp]; *fyuA* [776 bp]; *stxf2* [404 bp]; *hlyE* [616 bp]; *fimC* [496 bp]; eae [482 bp]; *bfp* [254 bp]. For each bacterial strain, a VGs profile is created by marking if certain gene is present or absent from genome of tested bacteria, as following: *filled box* presence of detected gene. *Open box* lack of detected gene. * *Citrobacter spp.* 303C and *Citrobacter spp.* PCM 1527 strains lacked tested virulence genes

database construction and searching software, which has revolutionized the detection and identification of bacteria [1, 12, 17].

Bacteria are characterized by significant diversity, and there are numerous examples of bacterial clones that belong to well-known species which display atypical traits. With their remarkable ability to fluctuate in metabolism activity, cell physiology, and genetic variability, bacteria can adapt to almost each niche both in host organisms and abiotic habitat. By horizontal transfer of genetic elements bacteria can acquire new genes and thus their genome can evolve, which is connected with changes of bacterial properties on both phenotypic and molecular level [2, 34]. In these cases, it is difficult to identify the examined bacterial strain even with the application of a highly advanced methodology based on new technologies. One method of bacteria identification can prove insufficient to obtain an undeniable and evident assignation of bacteria with an ambiguous phenotype to a genus or species level [2, 34].

This paper presents interesting research findings obtained during the identification of the bacterial strain of *Enterobacteriaceae* rods isolated from reptile feces. The

identification of isolated bacteria was conducted with conventional diagnostic media and molecular methods such as DNA-based assays and an innovative method such as MALDI-TOF MS Biotyper. It was observed that one strain of the intestine rods isolated from reptiles indexed as 305C displayed discrepant traits on preliminary diagnostic trail such as strains indexed as 305C biochemical profile with VITEK Compact 2 identification system and growth characteristic of diagnostic media typical of *E. coli* and colonies with mucoid surface that mucous morphology in general is characteristic trait of other enteric rods such *Klebsiella spp*. Microscopic observation of positive–negative staining proved that the bacterial strain 305C possesses a thin capsules. With the aim to determine what genus or species of *Enterobacteriaceae* family 305C strain with ambiguous phenotype belongs to, we have applied advanced identification methods tested strains was identified as *Citrobacter freundii* by mass spectrometry method with MALDI-TOF AUTOFLEX III SmartBean (BrukerDaltonics), as *Citrobacter braakii* with the species secure identification and as *E. coli* with secure genus identification. Verification of MALDI-TOF MS results for the 305C strain performed with a different model of mass spectrometer—UltraflExtreme (BrukerDaltonics), assigned 305C as *E. coli* with all score values to an acceptable level.

Further analysis including the application of VITEK Compact 2 identification system assigned the strain 305C with an ambiguous phenotype as an *E. coli* strain with excellent identification confidence and the probability of 99 %. A set of genetic analyses was also conducted based on PCR reactions detecting conserved genetic sequences specific to *E. coli* species such as *uidA* gene, regions of *16 SrRNA* and *16S-ITS-23SrRNA*. Results of all reactions confirmed that the 305C strain belongs to the *E. coli* species in terms of the composition of the genome isolated from the tested strain [41].

We have shown a diagnostic procedure of bacterial strain with ambiguous phenotype with broad choice of methods. Results of each method provide a significant data to the identification trial. Thanks to application of both conventional and molecular tests we obtain information about morphological properties of tested strain and as well as we determined unique profile of peptides and profile of genetic sequences specific to *E. coli* species. Analysis and comparison of all obtained results enables us to not only final identification atypical bacterial isolate as *E. coli*, but also precise examination and characterization of tested strain. We also have presented application and effectiveness of various methods in identification of microorganisms.

In current research both devices of MALDI-TOF vary in the type of laser source, a version of analyzing software and the MALDI-Biotyper library database version. It seems that a different type of ion source is the most important factor responsible for slightly discrepant identification results obtained for the 305C strain with two different devices. Despite the discrepancy in MALDI-TOF MS Biotyper identification of the 305C strain using both devices, other results of identification with biochemical (VITEK) and genetic (species-specific PCR) methods suggested that the tested strain belongs to the *E. coli* species. *Escherichia coli* and *Citrobacter spp.* belong to the same *Enterobacteriaceae* family and they are closely related. Most likely *Escherichia spp.* and *Citrobacter spp.* share similar proteins and with a slightly different ion fragmentation can give similar mass spectra [11]. Close relationship between *Escherichia spp.* and *Citrobacter spp.* could by supported by the fact that these bacteria can share the same or closely similar antigens. *Citrobacter braakii* isolated from an aquatic environment cross reacted with polyvalent *E. coli* O157 antiserum [22, 30]. As closely related bacterial strains *E. coli* and *Citrobacter* could have very similar mass spectra and largely share biochemical properties. These two species of *Enterobacteriaceae* family were difficult to differentiate when grown on an enriched bacterial growth medium such as Columbia Blood Agar [9]. In cases of *E. coli* and *Citrobacter spp*., especially of strains with an atypical phenotype or closely related ones, the application, of more specialized identification methods with higher discriminatory power is crucial.

Although MALDI-TOF MS Biotyper is an innovative and promising tool in the identification of bacteria, it also has its limitations, the most important of which is not sufficiently developed library of reference mass spectra as well as a potential influence of growth conditions of the analyzed microorganisms.

In the current study, it was proved that both databases included the same number of reference mass spectra—Mass Spectrum Profiles, so the limitation in the database is not the source of confusing identification of the tested strain 305C. According to literature [14, 32, 39], the reference library incorporated with MALDI-TOF MS Biotyper system could encounter microorganism isolates missing from the database, especially the microorganisms isolated from no typical niches. To reduce misleading identification results or cases when in the database, there are no reference mass spectrum matches to a tested isolate, and the mass spectra library should be continuously developed and broadened. Recently, there has been a suggestion to improve MALDI-TOF MS Biotyper platform accuracy by supplementing the mass spectra library with data about the *16S rRNA* sequence of the examined microorganisms [7].

Analysis of a microorganism's genome is still considered as a decisive and powerful method of microbial identification. Each bacterial species has a stable part of a genome with genes and genetic sequences strongly specific (about 10 % of the whole bacterial genome). These

conservative genetic sequences are used in molecular methods of bacteria identification. Other bacterial traits, such as biochemical properties, morphology of the microbial colony and even proteins analyzed, are products of genes expression and may yield post-translated modifications thus causing a diversity among bacteria [35].

Comparing the various methods that enable detection of microorganisms, especially isolates displaying ambiguous phenotypes or pathogenic bacteria, is significant for research of biodiversity of bacteria, examination of new bacterial species or isolates of microorganisms with an typical phenotype and also important to improve the diagnostics of bacterial diseases in clinical trial. Current paper provides important data of application different methods in identification of microorganisms. We have shown that in diagnostic procedure of bacterial strain displaying important is determination of morphological, physiological, and biochemical properties as well as examination of genome and proteome of tested bacterial isolate. The broad range of methods is use the higher probability of successful identification of examined bacterial strain, and the better discriminatory power is acquired in diagnostic procedure. In literature, there are other research comparing different assays in identification of microorganisms [26, 29, 32]. For example, methods applied in the cases of bacterial vaginosis (caused most often by *Gardnerella vaginalis*, *Atopobium vagina, Prevotella, Peptostreptococcus, and Bacteroides spp.*) revealed that in comparison to gram-staining of vaginal smears as a gold standard, qPCR had the highest sensitivity, accuracy, and negative predictive value [16]. Van Veen et al. [39] performed an analysis of 980 clinical isolates of bacteria and yeasts in which they showed that in terms of effectiveness, MALDI-TOF MS occurred to be a significantly more reliable method for accurate determination of a microbial genus or species than conventional biochemical assays.

Conclusion

In conclusion, the choice of identification method of bacteria has to be carefully analyzed. The kind and source of detected bacteria (environmental or clinical isolates) as well as the purpose of research (typically diagnostic or scientific) are of considerable significance. In presented paper, we have revealed that the identification of bacterial isolate displaying atypical traits is a complex procedure and requires an application more than one method. We have designed a identification procedure with a broad range of methods, such as colony morphology (macroscopic observation of bacterial growth on culture media), metabolism activity and biochemical properties (automated system of microorganisms identification VITEK Compact 2), analysis of bacterial proteome (MALDI-TOF MS Biotyper), and genetic analysis of bacteria (species- specific PCR reactions) to identify an isolate of Gram-negative rod with atypical phenotype. Application of both phenotypic (conventional) and molecular (with application of new technologies) methods significantly improve the discriminatory power of identification procedure and enables determination of genus or even species of microorganisms with atypical phenotype. Traditional phenotypic methods are useful in preliminary identification of bacteria and provide important data of the phenotypic or physiological traits for example presence of extracellular capsules. Molecular methods in general are more accurate and should be used as confirmatory tests for hard-to-identify or atypical isolates. MALDI-TOF MS is generally found as a very accurate, rapid, high-throughput proteomic technique for bacteria identification. DNA-based assays enable detection bacterial strains easily and directly from samples or from small amounts of cultured bacterial cells, thus improving the sensitivity and decreasing the time required for bacterial identification, at any level of specificity: strain, species, or genus. This paper is a great example of a screening research comparing application of various method in bacterial identification.

Acknowledgments The authors would like to thank Professor Andrzej Gamian (Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wroclaw, Poland) for the bacterial strains from the Polish Collection of Microorganisms; the director of the ZOO Wrocław, Radosław Ratajszczak; and employees of the Zoological Garden in Wrocław for their help in providing the biological samples and for the permission to use them in bacteriological research.

Funding The funding source(s) had no such involvement in presented research.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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