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Routine identification of *Campylobacter jejuni* and *Campylobacter coli* from human stool samples

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Abstract

Correct identification of *Campylobacter jejuni* and *Campylobacter coli* isolates to the species or subspecies level is a cumbersome but nevertheless important task for a routine diagnostic laboratory. The widely used biochemical tests might be often misleading while more sophisticated phenotypic or genotypic methods are not generally available. This investigation was performed to assess the performance of common biochemical identification in comparison with species-specific PCR and gas liquid chromatography of whole cell fatty acid extracts (GLC). A total of 150 consecutive isolates from human stool samples were investigated (134 *C. jejuni* ssp. *jejuni*, 14 *C. coli*, two *Helicobacter pullorum*). From these 144, 145 and 149 isolates were correctly identified by biochemistry, GLC and PCR, respectively. Biochemical identification of all *C. jejuni* isolates was confirmed by PCR. GLC detected both *H. pullorum* strains but misidentified two *C. coli* strains as *C. jejuni* and one *C. jejuni* strain as *C. coli*. No single method can be defined as 'gold standard' for identification of *C. jejuni* and *C. coli* but a combination of techniques is needed. Therefore a stepwise identification scheme starting with biochemical reactions is suggested. All results other than *C. jejuni* should be confirmed by further methods. For indoxyl acetate-positive isolates species-specific PCR is recommended while GLC seems to be advantageous in indoxyl acetate-negative isolates. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: *Campylobacter jejuni*; *Campylobacter coli*; Gas chromatography; Biochemical identification; Polymerase chain reaction

1. Introduction

Bacteria of the genus *Campylobacter* are the most frequent causative agents of acute bacterial enterocolitis worldwide [1]. *Campylobacter jejuni* and *Campylobacter coli* represent the most common species while others are rarely found like *C. lari*. The pathogenic role in gastrointestinal tract infections of other species like *C. hyointestinalis* is still under discussion. Catalase-negative species like *C. upsaliensis* or *C. concisus* require culture conditions different from the catalase-positive species and, apart from rare catalase-defective strains, do not cause particular problems in their differentiation from the catalase-positive species. Therefore this study focused on the catalase-positive strains isolated by a routine laboratory during a 12-month period. Identification of these microorganisms is a routine task for the clinical microbiologist, but correct differentiation to the spe-

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cies level is markedly hampered by the low biochemical reactiveness of these bacteria. Hence, over the last years, many phenotypic and genotypic methods have been proposed [2]. Biochemical differentiating schemes with up to 67 tests [3] and molecular techniques like polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) [4], DNA-DNA hybridization [5] or 16S rRNA gene sequencing [6] have been suggested. However, these procedures do not fully meet the needs of the daily routine laboratory, because they are cumbersome, costly and time-consuming. On the other hand, correct identification of *Campylobacter* sp. from human samples is required for epidemiological purposes. The integration of PCR methods in routine protocols of diagnostic laboratories as well as the availability of advanced phenotypic tests like the analysis of cellular fatty acid profiles [7] or pyrolysis mass spectrometry [8] therefore are promising attempts. This study was performed to develop a rapid, easy to perform, and reliable protocol for the correct identification of *C. jejuni* and *C. coli* isolates from human stool samples.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Campylobacter-like bacteria were isolated from human stool samples submitted to our laboratory for diagnostic purposes during a 12-month period between June 1995 and May 1996. All catalase-positive isolates were included in the study. The medium for primary isolation was blood-free Campylobacter agar base (Oxoid, UK) supplemented with 32 mg l^{-1} cefoperazone and 10 mg l^{-1} amphotericin B. Incubation in all experiments was carried out for 48 h at 37°C under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2). Subcultures were performed on non-selective agar plates (yeast-cysteine-agar) supplemented with 10% sheep erythrocytes.

2.2. Biochemical tests

Glucose fermentation, nitrate reduction and catalase were tested by routine laboratory procedures [9]. Oxidase test (Organon Teknika, Germany) and hippurate hydrolysis test (Rosco, Denmark) were performed according to the manufacturers' instructions. Indoxyl acetate hydrolysis was tested according to Mills and Gherna [10]. Antimicrobial susceptibility testing was performed using a disk diffusion test for nalidixic acid and cephalotin. No zone of growth inhibition was defined as 'resistant' while any zone of inhibition was defined as 'susceptible'.

2.3. Whole cell fatty acid analysis

Gas-liquid chromatography of cellular fatty acids was performed using a modification of the method described by Moss [11]. Briefly, bacteria were grown on Columbia agar plates (Columbia blood agar base No. 2, Difco, Germany) supplemented with 5% sheep erythrocytes. Growth from four agar plates was removed by scraping and suspended in a screw-capped tube containing 1 ml 0.7% $MgSO_4$. After centrifugation for 10 min at $300 \times g$ the cells were resuspended in 1 ml of 15% NaOH in 50% aqueous methanol and saponified at 100°C for 30 min. For methylation the sample was cooled to ambient temperature. Then 1 ml of hydrochloric acid-methanol reagent (325 ml of 6 N HCl, 275 ml methanol) and 1 ml of H_2SO_4-methanol reagent (325 ml 50% H_2SO_4, 275 ml methanol) were added and incubated at 80°C for 10 min. The resulting fatty acid methyl esters were extracted with 1.25 ml of a 1:1 mixture of butyl-methyl-ether and normal hexane (v/v) and washed with 3 ml of a NaOH/NaCl solution (0.3 M NaOH, 4 M NaCl). The samples were analyzed on a 25 m $\times$ 0.2 mm (inside diameter) fused-silica capillary column using a HP 5890 Series II gas chromatograph equipped with a flame-ionization detector (Hewlett Packard, USA). The column was temperature programmed from 170°C to 270°C at 5°C min^{-1} and was maintained at 270°C for 2 min. The injector temperature was 250°C and the detector temperature was 300°C. Ultrapure hydrogen was used as carrier gas. The fatty acid methyl esters were identified by comparing retention times to known standards (MIDI calibration standard mix, Hewlett Packard, USA). A model 3392A integrator (Hewlett Packard, USA) was used for quantification of peak areas. Analysis of the data was performed using the CLIN database of the MIS Microbial Identification System software (Microbial ID, USA).

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2.4. PCR

Bacterial DNA was prepared by suspending one colony in 1 ml of distilled water and heating for 10 min at 95°C. After centrifugation at 3000×*g* for 10 min 5.0 μl of the supernatant was added to the reaction mixture. For the reaction mixture (50 μl final volume) Taq DNA polymerase buffer (Pharmacia, Sweden) with 1 mM $MgCl_2$ was used. Per reaction, 1.5 U of Taq DNA polymerase (Pharmacia, Sweden) were added. PCR involved 27 cycles of consecutive denaturation (1 min, 95°C), primer annealing (1 min, 54°C), and chain extension (1 min, 72°C). Prior to cycling, the samples were heated at 95°C for 2 min. Finally, an additional extension step (5 min, 72°C) was performed. A Perkin Elmer Cetus GeneAmp System 9600 thermocycler (Perkin Elmer, USA) was used in all experiments. Primers (25 pmol in all experiments) THERM3/JEJ1/JEJ2, THERM1/COLI, THERM1/LARI [12], 69ar/FET1 [13] and FPH16/RPH16 [14] specific for *C. jejuni*, *C. coli*, *C. lari*, *C. fetus* and *Helicobacter pullorum*, respectively, were used. PCR products were separated on 1% agarose gels and stained with ethidium bromide.

2.5. DNA sequencing

PCR products were sequenced using a PRISM ready reaction dideoxy terminator cycle sequencing kit (Applied Biosystems, USA). The reaction mixture was subjected to 25 cycles consisting of a denaturing step (1 s, 98°C), primer annealing (15 s, 55°C) and chain extension (4 min, 60°C). PCR products were purified by gel filtration on S-200 HR columns (Pharmacia, Sweden), applied on a 6% polyacrylamide gel and analyzed on a 373A laser fluorescence sequencing system (Applied Biosystems, USA). Data were analyzed using the FASTA software on the EMBL database.

2.6. Definition of correct species identification

Since no 'gold standard' of *Campylobacter* identification is defined, the result of a synopsis of species-specific PCR with the phenotypic methods was referred to as correct identification.

3. Results

A total of 150 consecutive clinical isolates of putative *Campylobacter* sp. from different patients were included in this study. From these 134 isolates were *C. jejuni*, 14 were *C. coli* and two were *H. pullorum*.

3.1. Biochemical identification

All isolates were positive for oxidase and catalase activity and reduced nitrate. They did not utilize glucose. Of all strains 130 were indoxyl acetate- and hippurate-positive and therefore designated *C. jejuni* ssp. *jejuni*. Eighteen strains were indoxyl acetate-positive and hippurate-negative and thus designated *C. coli*. Two isolates were indoxyl acetate- and hippurate-negative, grew at 42°C but not at 25°C and were resistant to nalidixic acid. Therefore they were designated *C. lari*.

3.2. PCR

Using species-specific PCR 133 isolates yielded a product with primers specific for *C. jejuni* but were negative with primers for *C. coli*. Therefore they were determined to be *C. jejuni*. The amplicons of 100 of these strains were approximately 750 bp in size whereas the amplicons of 33 strains yielded a size of approximately 600 bp, which both were not within the expected range. Therefore products from two different strains were sequenced and the exact length was found to be 743 bp and 586 bp, respectively. Fifteen isolates revealed a product with primers specific for *C. coli* and simultaneously were negative with the *C. jejuni*-specific primers. Thus they were determined to be *C. coli*. All amplicons had a size of approximately 350 bp, which also was not within the expected range. Here sequencing revealed an exact length of 347 bp. The two isolates biochemically determined as *C. lari* were negative in the PCRs specific for *C. lari*, *C. jejuni*, *C. coli* and *C. fetus* but positive with primers specific for *H. pullorum*. This identification was confirmed by partial sequencing of the 16S rRNA gene, which revealed a homology of 99.7% with *H. pullorum*. No single strain yielded a product with more than one distinct primer pair.

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3.3. Gas liquid chromatography

By gas liquid chromatography of whole cell fatty acid extracts (GLC) 135 isolates were identified as *C. jejuni* and 13 isolates as *C. coli*. The two isolates determined as *H. pullorum* by species-specific PCR came out as *Oligella urethralis* with low identification scores.

3.4. Comparison of biochemistry, PCR and GLC

For 140 isolates the results of all three identification methods were consistent. Four isolates, biochemically identified as *C. coli*, were characterized as *C. jejuni* by PCR and GLC. Two isolates were determined primarily as *C. jejuni* by GLC while biochemically as well as by PCR they came out as *C. coli*. One isolate revealed a GLC pattern characteristic for *C. coli*, but was biochemically and by PCR identified as *C. jejuni*. One isolate came out as *C. jejuni* by biochemistry as well as by GLC, however, it yielded a PCR product with *C. coli*-specific primers, but not with *C. jejuni*-specific primers. Interestingly, sequencing of approximately 200 bp of the 16S rRNA gene of this strain revealed 98% homology with *C. jejuni* while the homology with *C. coli* reached only 93%. DNA sequencing of the primer annealing site in the 23S rRNA gene showed a point mutation from A to C in position 1885. The two strains determined to be *C. lari* biochemically and to be presumptive *O. urethralis* by GLC were identified as *H. pullorum* by PCR.

4. Discussion

Correct identification of *C. jejuni* and *C. coli* isolates from human stool samples is needed rather for epidemiological purposes than for therapeutic consequences. Routine laboratories in particular, which usually isolate *Campylobacter* strains more often than specialized centers, however, should be able to perform the identification in order to detect changes in epidemiological as well as in antibiotic resistance patterns. Furthermore, the sudden increase of a normally rare *Campylobacter* species might help to detect otherwise unidentified outbreaks. On the other hand, rapid and simple differentiation of *Campylobacter* species in the routine laboratory is markedly hampered by the relatively slow-growing, fastidious and biochemically unreactive organisms. Advanced techniques like protein profiling, PCR, DNA-DNA hybridization, GLC or pyrolysis mass spectrometry could overcome these difficulties. However, to expect a laboratory isolating hundreds of strains a year to apply these methods routinely seems to be unrealistic. Therefore the aim of this study was to develop a simple stepwise procedure for the reliable identification of the most frequent *Campylobacter* spp. isolated from human stool samples.

Biochemical tests are the basis of *Campylobacter* identification. They commonly are cheap, rapid, easy to perform and are adopted in almost all laboratories involved in the identification of *Campylobacter* sp. The main disadvantages of these tests are their relatively low discriminatory power particularly for rare *Campylobacter* sp. and, in addition, the instability of phenotypic markers. In this study we applied six often used biochemical markers, namely oxidase, catalase, nitrate reduction, glucose utilization, indoxyl acetate hydrolysis and hippurate hydrolysis. Applying these tests, 144 of 150 isolates were identified correctly. Four strains biochemically determined as *C. coli* proved to be hippurate-negative *C. jejuni* by PCR and GLC. The finding of such strains is consistent with previous reports [15]. Two strains determined as *C. lari* proved to be *H. pullorum* by genotypic methods. There are no biochemical markers available for the discrimination of *C. lari* and *H. pullorum* [16] and therefore even an extended panel of biochemical reactions would not achieve this differentiation.

It is interesting to note that all indoxyl acetate- and hippurate-positive isolates proved to be *C. jejuni* also by the other techniques. *C. jejuni* is by far the most frequently isolated species. Therefore we suggest biochemical differentiation as the first step in the identification of catalase-positive *Campylobacter* species. Biochemical differentiation is suitable to confirm the preliminary identification of the isolate as *Campylobacter* sp. as well as to correctly identify hippurate-positive *C. jejuni* which represented 87% of all isolates in our study. For all other, i.e. hippurate-negative, isolates, an additional identification technique should be applied.

One promising attempt for this purpose seems to

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be GLC of whole cell fatty acid extracts. This technique has been successfully used for the identification of various fastidious organisms [11,17]. Its effectiveness and reliability have been repeatedly demonstrated also for *Campylobacter* spp. [7,18]. In the present study 135 isolates were designated *C. jejuni* by GLC, 132 of them correctly, including four hippurate-negative *C. jejuni* strains. This demonstrates the superiority of the method in this respect compared with biochemistry. On the other hand, both isolates of *H. pullorum* were falsely determined as *O. urethralis*. However, the reason for this misidentification was the lack of an evaluated fatty acid profile of *H. pullorum* in the MIS database. Meanwhile a series of *H. pullorum* isolates has been analyzed by GLC and the mean profile has been added to the database [19]. Using the GLC technique, 13 isolates were determined as *C. coli*, 12 of them correctly. Therefore, according to previous studies [7] GLC proved to be valid for identification of the most common *Campylobacter* spp. Advantages of the method are that GLC patterns are produced in every case, which may lead to the identification of *Campylobacter*-like isolates also in the case of an unexpected species, e.g. *H. pullorum*. On the other hand, two *C. coli* isolates were misidentified as *C. jejuni* by GLC while one *C. jejuni* isolate was misidentified as *C. coli*. This underlines a distinct drawback of the method concerning the discrimination of these two species. This is also illustrated by the classification of *C. jejuni* and *C. coli* in a common GLC group by other authors [7]. We therefore recommend GLC as a valuable secondary technique for isolates that are not suspected to be *C. coli* or *C. jejuni*.

Using species-specific PCR, all *C. coli* strains and the two *H. pullorum* strains were identified correctly. One strain identified as *C. jejuni* by biochemical tests as well as by GLC yielded a product of the expected size with *C. coli*-specific primers but not with *C. jejuni*-specific primers. Partial sequencing of the 16S rRNA gene revealed a higher homology with *C. jejuni* than with *C. coli*. Obviously this finding has to be interpreted cautiously since homology between the entire 16S rRNA genes of both species is very high [5]. However, in connection with positive hippurate hydrolysis and GLC results this isolate was identified as *C. jejuni*. A point mutation in the primer annealing site of the 23S rRNA gene could be detected, which might have been the reason for the false positive result of the *C. coli*-specific PCR and the simultaneously negative *C. jejuni*-specific PCR. This finding on one hand demonstrates the high specificity of the employed PCR system. On the other hand it underlines the fact that a single PCR assay might not be sufficient for a correct species identification but should be combined with complementary, e.g. phenotypic or hybridization, techniques. Concerning the specific PCR for *C. jejuni* according to Eyers et al. [12] we found several strains carrying the internal transcribed spacer within the 23S rRNA gene. In this study they amounted to one third of the strains. However, the size of this spacer as well as the size of the *C. jejuni*- and *C. coli*-specific products differed from the description of Eyers et al. [12]. While they described sizes of 710/810 bp, 390 bp and 290 bp, we found 586/743 bp, 347 bp and 223 bp for *C. jejuni*-, *C. coli*- and thermophilic *Campylobacter* sp.-specific products, respectively. Nevertheless, PCR is a reliable identification method for *Campylobacter* spp., in contrast to GLC also for discrimination between *C. jejuni* and *C. coli*. A major practical disadvantage is the relatively high cost for the identification of a single isolate. In the case of a questionable *C. jejuni*/*C. coli* for example, at least six assays have to be performed: the isolate with *C. jejuni*- and *C. coli*-specific primers, two negative and two positive controls. Thus PCR in the routine laboratory might be performed as a batch procedure, e.g. every 2 weeks.

In conclusion, we suggest a stepwise scheme for the identification of *C. jejuni* and *C. coli* in a routine laboratory. Basically a range of biochemical reactions should be checked for (i) confirmation of the suspected genus and (ii) definitive identification of *C. jejuni* isolates by a positive hippurate test. For all other, i.e. hippurate-negative, isolates a second identification step should be applied. In the case of species positive for indoxyl acetate hydrolysis, specific PCR should be performed while in other isolates GLC of whole cell fatty acid extracts might be superior. In the case of an isolate not identified by this scheme the strain could be referred to a specialized or reference laboratory where identification can be achieved by further phenotypic or genotypic techniques.

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