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Discrimination of *Helicobacter pullorum* and *Campylobacter lari* by analysis of whole cell fatty acid extracts

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Abstract

Helicobacter pullorum and *Campylobacter lari* are rarely isolated from humans with acute enteritis. Hitherto the two species could only be identified by genotypic techniques. Gas liquid chromatography of whole cell fatty acid extracts is described as the first phenotypic method for discrimination of the two species. Cholesteryl glucoside, a characteristic feature of the genus *Helicobacter*, but seldom found in other bacteria, could not be detected in *Helicobacter pullorum*. Therefore, rapid determination of this glycolipid may serve as a discrimination marker for *Helicobacter pullorum* from most other *Helicobacter* species.

Keywords: *Helicobacter*; *Campylobacter*; Gas chromatography; Cholesteryl glucoside; Fatty acid

1. Introduction

Campylobacter lari is a rare, but well-known human pathogen. It may be isolated from animals and environmental sources like sea water and can produce acute diarrheal illness in normal hosts as well as bacteremia in immunocompromised patients [1]. *Helicobacter pullorum*, on the other hand, has only recently been described [2]. It is frequently isolated from poultry, whereas its role as a human pathogen is still under discussion.

Identification to the species level in general is difficult in campylobacters due to the availability of only a few biochemical reactions. Especially for the discrimination of *C. lari* from *H. pullorum*, no suitable phenotypic marker exists [3]. Therefore, reliable identification of the two species can only be achieved by genotypic techniques like species specific PCR [2,4] or DNA–DNA hybridization [2].

Gas liquid chromatography of whole cell fatty acid extracts (GLC) is a well established identification method for a variety of Gram-positive and Gram-negative bacteria. It was also successfully used for differentiation of *Campylobacter* sp. [5]. The presence of cholesteryl glucoside (CG), on the other hand, has been described as characteristic feature of *Helicobacter* sp. [6], while, to our knowledge it has not been investigated in *Campylobacter* sp. This study was performed to assess whether analysis of whole cell fatty acid composition and detection of CG would be appropriate phenotypic techniques for discrimination of *C. lari* and *H. pullorum*.

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2. Materials and methods

2.1. Bacterial strains and culturing methods

Seventeen strains of *H. pullorum* and *C. lari*, respectively, were investigated with GLC. *H. pullorum* strains NCTC 12827, NZ 6350-92, NZ 3166-93, NZ 3659-93, NZ 1385-94, NZ 5062-94, FR95/7233, FR96/2903 isolated from humans and H 154-CS2, NZ 459-94, NZ 1903-94, NZ 3758-94, NZ 4910-92, NZ 4914-92, NZ 5381-92, NZ 2264-95 and NZ 2357-95 isolated from poultry were studied. *C. lari* strains from humans (NZ 3851-91, NZ 878-93, NZ 3117-93, NZ 145-95, NZ 4252-95, LMG 7607, LMG 9887, LMG 9888, LMG 9889, LMG 9914, LMG 11251, LMG 11760, CCUG 15031, CCUG 19512), dog (C 751-92), horse (LMG 9152) and sea water (LMG 8844) were investigated. For the detection of cholesteryl glucoside, *H. pylori* ATCC 43504 was used as positive control and compared with *H. pullorum* strains NCTC 12827, FR95/7233, FR96/2903, NZ 4910-92, NZ 4914-92, *C. lari* NCTC 11352, *C. jejuni* NCTC 11351, *C. coli* NCTC 11366 and *C. fetus* NCTC 10842. For GLC bacteria were grown on Columbia agar plates supplemented with 5% sheep erythrocytes. For detection of CG, bacteria were grown on yeast–cystein agar supplemented with 10% human erythrocytes and 10% horse serum. Plates were incubated at 37°C for 48 h under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2).

2.2. Gas liquid chromatography of whole cell fatty acid extracts (GLC)

Fatty acids were extracted in a modification of the method described by Miller [7]. Briefly, colonies from four agar plates were harvested by scraping and suspended in a screw-capped tube containing 1 ml 0.7% aqueous $MgSO_4$. 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, 1 ml of hydrochloride–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 the mixture 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 *n*-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 by 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, CA, USA). The column was temperature programmed from 170°C to 270°C at 5°C/min 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 a carrier gas. The fatty acid methyl esters were identified by comparing retention times to a known standard (MIDI calibration standard mix, Hewlett Packard, CA, USA). A model 3392A integrator (Hewlett Packard, CA, USA) was used for quantitation of peak areas. Data were analyzed using the Microbial Identification System software (MIDI, DE, USA).

2.3. Detection of cholesteryl glucoside (CG)

Rapid detection of CG by thin layer chromatography was performed in a modification of the method described by Matsuyama [8] et al. and Haque et al. [6]. Briefly, bacterial mass was suspended in 400 μl of chloroform–methanol (2:1, v/v). The suspension was applied on a Silica Gel 60 plate (Merck, Darmstadt, Germany), air dried and predeveloped up to one-third of the plate in chloroform–methanol (2:1, v/v). After drying the plates were redeveloped in chloroform–methanol–water (70:30:5, v/v/v). The plate was then sprayed with 0.2% orcinol in 2 N sulfuric acid and heated at 120°C until a purple-red color appeared.

3. Results

By analysis of whole cell fatty acid extracts 16 of 17 *C. lari* strains were correctly identified by the MIS software (CLIN database, identification score from 0.304 to 0.904). One strain (CCUG 19512) was repeatedly misidentified as *Pseudomonas diminuta*. Since *H. pullorum* is not represented in the MIS software database, isolates of this species were not recognized. Highest similarity was found with the fatty acid profile of *Oligella urethralis* in 16 strains and

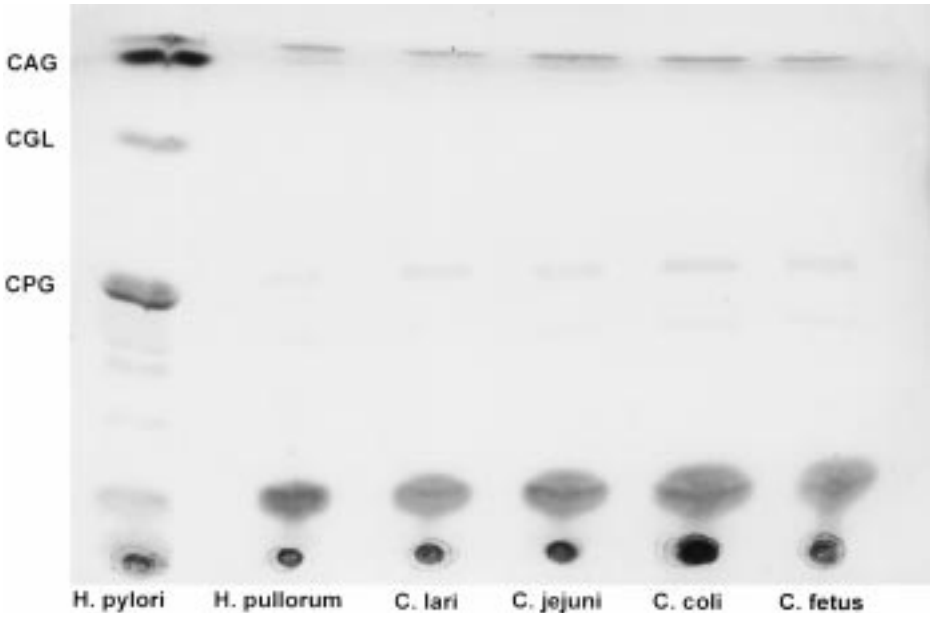

Fig. 1. Direct colony thin layer chromatography of *Helicobacter* spp. and *Campylobacter* spp. CAG, cholesteryl acyl glucoside; CGL, cholesteryl glucoside; CPG, cholesteryl phosphatidyl glucoside.

Oligella ureolytica in one strain (NZ 5381-92) (identification score from 0.235 to 0.670). Two fatty acids differed quantitatively between the two species (18:1 ω7c 46.2 ± 7.0 vs. 54.8 ± 2.3; 16:1 ω7c 8.0 ± 3.8 vs. 0.2 ± 0.3 for *C. lari* and *H. pullorum*, respectively; all data given in percent of whole fatty acids, mean ± S.D.). The corresponding ranges were: 18:1 ω7c 34.1–60.9 vs. 51.8–60.3; 16:1 ω7c 1.4–17.6 vs. 0.0–1.1 for *C. lari* and *H. pullorum*, respectively (data given in percent of whole fatty acids). Three *C. lari* strains exhibited a higher portion of 18:1 ω7c fatty acid than the *H. pullorum* strain with the lowest 18:1 ω7c. The 16:0 3OH fatty acid was detected in 16 of 17 *H. pullorum* strains (3.7 ± 0.3% whole fatty acids) with the exception of strain NZ 3659-93 while all 17 *C. lari* strains were negative for this fatty acid. Additionally, significant amounts of a fatty acid with a retention time between 14:0 and 15:0 chain length was detected in 15 of 17 *H. pullorum* strains (1.1 ± 0.1% of whole fatty acids). Strains FR95/7233 and FR96/2903 were negative for this fatty acid. However, this fatty acid could not be identified by comparison with the standard. The results are shown in Table 1.

The presence of cholesteryl glucoside in *H. pylori* as described by Hirai [9] was confirmed. R_f values were approximately 0.9, 0.8 and 0.5 for cholesteryl acyl glucoside (CAG), cholesteryl glucoside (CGL) and cholesteryl phosphatidyl glucoside (CPG), respectively. Cholesteryl glucoside could neither be detected in *H. pullorum* nor in C. *lari*, *C. jejuni*, *C. coli* and *C. fetus* (Fig. 1).

Table 1
Cellular fatty acid profiles of *Campylobacter lari* and *Helicobacter pullorum*

Species	No. of strains	14:0	Unidentified	14:0 3OH	16:0	16:0 3OH	16:1 ω7c	18:0	18:1 ω7c
Campylobacter lari	17	5.2 ± 2.3	0	4.4 ± 0.9	34.1 ± 3.5	0	8.0 ± 3.8	1.5 ± 1.0	46.2 ± 7.0
Helicobacter pullorum	17	3.6 ± 0.4	1.0 ± 0.4	3.6 ± 0.2	28.5 ± 1.8	3.7 ± 0.3	0.2 ± 0.3	2.8 ± 0.7	54.8 ± 2.3

Values are given as percentages of total fatty acids, arithmetic means ± S.D.; fatty acids below 0.6% of total fatty acids in both species are omitted.

4. Discussion

Fatty acid profiles of *C. lari* and *H. pullorum* share some main characteristics, such as the preponderance of 16:0 and of 18:1 fatty acid as well as the absence of cyclic fatty acids. However, quantitative differences for two fatty acids, namely 16:1 ω7c and 18:1 ω7c were detected. Additionally, all *C. lari* strains lacked the 16:0 3OH fatty acid present in all but one *H. pullorum* strains. Though there was an overlap for the differing fatty acids in single strains at least two of the three fatty acid features were characteristic in every strain. These results show that gas liquid chromatography of whole cell fatty acids is an appropriate phenotypic method for discrimination of *C. lari* and *H. pullorum*. The presence of an additional, unidentified fatty acid eluting with a retention time characteristic for saturated fatty acids with chain lengths between 14 and 15 carbon atoms is characteristic for *H. pullorum* and was found in 89% of the isolates.

The presence of CG is a characteristic feature of the genus *Helicobacter* with *H. pametensis* and *H. cinaedi* being the only two species lacking this group of lipids [6,10], while *H. pullorum* has not been investigated so far. Among other prokaryotes, CG has only been detected in mycoplasmas, spiroplasmas, acholeplasmas and spirochetes [6]. Since detection of CG by direct thin layer chromatography is a rapid and easy to perform technique, it is well suited for the discrimination of biochemically inert bacteria. In the present study, several species of the genus *Campylobacter* were negative for CG as expected. This glycolipid was also notably absent in *H. pullorum*. Thus *H. pullorum* together with *H. cinaedi* and *H. pametensis* are the only *Helicobacter* sp. negative for CG. Therefore, detection of CG is a useful marker for rapid discrimination of these three intestinal *Helicobacter* species from the majority of other *Helicobacter* sp. with a gastric habitat. Thus two simple and rapid methods for the analysis of cellular fatty acids permit the discrimination of *H. pullorum* from related campylobacters and helicobacters as well.

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