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Characterization of spirochetal isolates from arthropods collected in South Moravia, Czech Republic, using fatty acid methyl esters analysis

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Abstract

Aim of this study was to evaluate cellular fatty acid analysis for characterization of spirochetes. Strains were isolated from arthropods collected in South Moravia, Czech Republic. Fatty acid methyl esters (FAME) profile was determined for five *Borrelia burgdorferi* sensu lato (s.l.) strains isolated from *Ixodes ricinus* ticks, one "*Spironema culicis*" strain recovered from mosquito *Culex pipiens* and seven spirochetal strains (not identified yet) isolated from mosquitoes and blackflies. Analysis was performed using a gas chromatography column in conjunction with Microbial Identification System Sherlock (MIDI Inc., Newark, DE, USA). Results obtained on the basis of cluster analysis of FAME profiles showed, that the *B. burgdorferi* sensu lato isolates could be well separated from other spirochetal isolates. We recommended method used in this study as a useful tool for preliminary identification of spirochetes isolated from ticks and dipterans.
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1. Introduction

Spirochetes comprise several genera. They were found free in marine and fresh water, or in their vertebrate and arthropod hosts [1]. *Borrelia burgdorferi* sensu lato (s.l.), the causative agent of Lyme disease, is transmitted through the bite of infected ixodid ticks (in the Czech Republic *Ixodes ricinus* ticks) [2,3]. Complex *B. burgdorferi* s.l. includes at least 10 different borrelial genospecies, but only *B. burgdorferi* sensu stricto (s.s.), *B. garinii* and *B. afzelii* have been identified as pathogenic for humans [4]. Because of importance of the disease, many further studies were done to prove, that not only *B. burgdorferi* s.l. could be found in ixodid ticks and that different spirochetes can survive not only in ixodid ticks [5–9].

Spirochetes associated with the blood-sucking insects were observed in midgut, malphigian tubules and salivary glands of tabanid flies [5,6], blackflies [7] and mosquitoes [8,9]. In South Moravia, Czech Republic, the spirochetes were detected in blood-sucking insects of the family *Culicidae* [9–11] and *Simuliidae* [12]. The spirochetes were found in all developmental stages (except for eggs) of mosquitoes and blackflies and were observed in overwintering mosquitoes from the beginning through the end of winter period [12]. The pathogenicity of new spirochetal strains is still unknown.

Spirochetal cells have high lipid content [13]. In *B. burgdorferi* s.l. lipids comprise about 25–30% of the cell dry weight, especially phospholipids and glycolipids with different fatty acid compositions [14]. The occurrence and relative amount of each component is a stable indication of the phenotype when the bacteria are grown under standard cultivation conditions [15,16]. Fatty acid metabolism is directed by the chromosome and is not known to be under plasmid control. The presence of certain fatty acids has been shown to correlate with taxonomic conventions [17,18].

Cellular fatty acid analysis is widely used for characterization of many bacterial species [16,19,20]. The Microbial

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Identification System (MIS) Sherlock produced by MIDI Inc. (Newark, DE, USA) is a well-established identification method for a variety of Gram- and Gram-positive bacteria [21]. The identification of *B. burgdorferi* s.l. strains is often difficult [4], therefore we decided to evaluate FAME analysis for the identification of new spirochetal isolates.

2. Materials and methods

2.1. Chemicals

The components of cultivation media BSK-H medium, rifampicin and phosphomycin were purchased from Sigma (St. Louis, MO, USA), rabbit serum from Gibco (Carlsbad, CA, USA), phosphate buffered saline (Dulbecco A) from Oxoid (Basingstoke, UK) and magnesium sulphate from Pliva-Lachema (Brno, Czech Republic). Chemicals (methanol, sodium hydroxide, hydrochloric acid, hexane, methyl-*tert*-butyl ether) used for fatty acid methyl esters preparation originated at Merck (Darmstadt, Germany).

2.2. Bacterial strains

Five *B. burgdorferi* s.l. isolates and seven non-identified spirochetes were tested. Tick-isolates, which belonged to the *B. burgdorferi* s.l. group were confirmed as a *Borrelia* spp. and identified to the species level according Postic [22]. Spirochetes isolated from dipterans allowed no bands characteristic for *Borrelia* spp. [22] and were therefore assigned as non-identified. In the study was also included a new spirochetal strain "*Spironema culicis*" (it has not yet been formally named) derived from mosquitoes [23]. To compare validity of the fatty acid analysis *B. burgdorferi* s.l. type strains were chosen (Table 1).

2.3. Culture conditions

All strains were cultivated in BSK-H medium with the supplement of rabbit serum (6%) and antibiotics rifampicin (50 μg/ml) and phosphomycin (100 μg/ml). Bacteria were incubated at 33 °C for the period needed to generate a cell density of 10^8 organisms ml^{-1} and checked via dark-field microscopy for purity and concentration before they were harvested. Spirochetes were harvested by centrifugation at 8500 × *g* for 30 min. The cells were washed twice in phosphate-saline buffer (PBS) and then twice 0.7% aqueous $MgSO_4$.

2.4. Extraction of cellular fatty acids

After centrifugation, the whole cells (about 20 mg wet weight) were transferred into tubes with Teflon-lined screw caps, converted to the methyl esters and extracted. Saponification, methylation and extraction of the cellular fatty acids were performed according to the operating manual of the MIS Sherlock (MIDI Inc., Newark, DE, USA) [21]. Briefly, 1.0 ml of reagent 1 (45 g sodium hydroxide, 150 ml methanol and 150 ml distilled water) is added to tube with cells. The tubes are vortexed and heated in boiling water bath for 30 min. The tubes are cooled and 2 ml of reagent 2 (325 ml 6.0 N HCl and 275 ml methanol) is added. After vortexing, the tubes are heated for 10 min at 80 °C. Addition of 1.25 ml of reagent 3 (200 ml hexane and 200 ml *tert*-butyl ether) is followed by recapping and gentle tumbling on a rotator about 10 min. Next, the aqueous phase is pippeted out and 3 ml of reagent 4 (10.8 g NaOH in 900 ml distilled water) is added to the organic phase and the tubes are tumbled for 5 min. About 2/3 of the organic phase is pippeted into GC vial which is ready for analysis.

Table 1
Sources of the spirochetes used in this study

Strain	Source/country	Species designation
B31	*Ixodes scapularis*/USA	*B. burgdorferi* sensu stricto (type strain)
BR194	*Ixodes ricinus*/Czech Republic	*B. burgdorferi* sensu stricto
20047	*Ixodes ricinus*/France	*B. garinii* (type strain)
BR14	*Ixodes ricinus*/Czech Republic	*B. garinii*
BR92	*Ixodes ricinus*/Czech Republic	*B. garinii*
BR122	*Ixodes ricinus*/Czech Republic	B. garinii
BR132	*Ixodes ricinus*/Czech Republic	*B. garinii*
VS461	*Ixodes ricinus*/Switzerland	*B. afzelii* (type strain)
VS116	*Ixodes ricinus*/Switzerland	*B. valaisiana* (type strain)
BR91	*Culex* (*Culex*) *pipiens*/Czech Republic	"*Spironema culicis*"
BR116	*Culex* (*Culex*) *pipiens*/Czech Republic	Not identified
BR134	*Culex* (*Culex*) *pipiens*/Czech Republic	Not identified
BR135	*Culex* (*Culex*) *pipiens*/Czech Republic	Not identified
BR151	*Culex* (*Culex*) *pipiens*/Czech Republic	Not identified
BR173	*Culex* (*Culex*) *pipiens*/Czech Republic	Not identified
BR208	*Culex* (*Culex*) *pipiens*/Czech Republic	Not identified
BR231	*Simulium* (*Simulium*) *noelleri*/Czech Republic	Not identified

2.5. Gas chromatography

The samples were analyzed on 25 m by 0.2 mm phenyl–methyl siloxane capillary column using a HP 6890 gas chromatograph equipped with flame-ionization detector. The gas flow rates were approximately 400 ml/min for air, 30 ml/min for hydrogen and 30 ml/min for nitrogen. The temperatures used were 250 °C for the injection port and 300 °C for the detector. After injection, the oven temperature was increased from 170 to 270 °C at a rate of 5 °C/min, held at 310 °C for 2 min, and then returned to 170 °C before the next sample was injected. The fatty acid methyl esters were compared to a known standard (MIDI calibration standard mix).

2.6. Peak naming and cluster analysis

MIS software was used to identify the peaks by retention time, to determine their area and to calculate the equivalent chain length (ECL). Peaks were named using the TSBA40 library parameters. The automated communication process exist between ChemStation software (Agilent Technologies), which commanded the gas chromatograph and Sherlock software (Sherlock Library Generation Software, MIDI Inc., Newark, DE, USA). Briefly, GC parameters are downloaded from the Sherlock (method TSBA40) to the ChemStation. The peak retention time, width, and area data from the integrator are transmitted and processed by the ChemStation. Raw chromatographic data are transferred back to the Sherlock, the peaks are identified by equivalent chain length value.

To compare obtained FAME profiles, cluster analysis was used to create a dendrogram Yielded qualitatively and quantitatively reproducible fatty acid composition profiles were used for futher dendrogram cluster analysis technique produced by unweighted pair matchings by Sherlock's application (Sherlock Library Generation) [21b].

3. Results and discussion

The major indicators of microorganisms are fatty acids of 10–18 carbon atoms [24]. According to Brondz [25], fatty acid analysis could play a significant role also in the classification of spirochetes. We confirmed like Moore [20] that FAME analysis provides a reasonable, rapid and cost-effective alternative method.

For an effective typing system based on comparison of cellular fatty acid profiles, all parts of the experiment must be carefully standardized. The incubation step of the FAME protocol is the crucial part of this technique. In this study, fatty acid extraction and factors such as media, incubation times, temperature, and chromatographic conditions were standardized for all seventeen spirochetal strains.

The FAME profiles were successfully evaluated in all tested spirochetal strains. At least 38 different fatty acids were detected, their frequency was compared between three different groups according to the characterization results—*B. burgdorferi* s.l., non-identified spirochetes and "*S. culicis*" (Table 2). Eight fatty acids were detected in all strains. These fatty acids were the most common ones and may therefore represent the qualitative fatty acid pattern of spirochetes ($C_{12:0}$, $C_{14:0}$, $C_{15:0}$, $C_{16:0}$, $C_{18:0}$, $C_{18:1}$ ω9c, summed feature 3 and summed feature 5).

All *B. burgdorferi* s.l. strains share some main characteristics, such as the predominance of $C_{16:0}$ (39–53%), followed by $C_{18:1}$ ω9c (24–34%) and $C_{18:0}$ (5–10%) as well as the low content of cyclic fatty acids (Table 3), which is in acceptance with previous studies of *B. burgdorferi* s.l. [13,14,26–28]. As the predominant fatty acid $C_{16:0}$ was described also in the genera *Treponema* [13,28,29], *Brachyspira* (*Serpulina*) [13,26] and *Leptospira* [13,26].

The major fatty acids of six isolates of non-identified spirochetes from dipterans were $C_{16:0}$ (27–40%), $C_{12:0}$ (11–30%), $C_{18:1}$ ω9c (6–13%) and $C_{18:1}$ ω7c (2–11%). The dominance of $C_{12:0}$ can differentiate non-identified spirochetes from other *B. burgdorferi* s.l. strains. $C_{18:0}$ fatty acid was significantly lower than in borrelial strains, and the same is valid for "*S. culicis*".

The strain BR91, proposed as a new species "*S. culicis*", appeared the most different among all analyzed spirochetes. The fatty acid composition was unique. Only in this strain, the $C_{14:0}$ was found to be the predominant fatty acid (42.4%) (Table 3). Other fatty acids, $C_{18:1}$ ω9c (9.52%) and $C_{18:0}$ (4.69%), had a similar incidence as strains isolated from dipterans.

Interestingly, differences in fatty acid composition between single isolates from ticks and dipterans also appeared. The $C_{13:1}$ AT12-13 fatty acid and summed features 1 and 2 ($C_{13:0}$ 3OH and/or $C_{15:1}$ iI/H, $C_{16:1}$ iso I and/or $C_{14:0}$ 3OH) were detected only in strains isolated from dipterans (non-identified spirochetes and "*S. culicis*") while all nine *B. burgdorferi* s.l. strains isolated from ticks were negative for these fatty acids. Difference in fatty acid composition could be shown in their vectors. The vector of *B. burgdorferi* s.l. in Europe is the tick *I. ricinus* [2], whereas the other analyzed spirochetes originated from dipterans species *Culex pipiens* and *Simulium noelleri.* The spirochetes are host-specific, it is known that some interactions between spirochetes and the host environment exist [30]. The visceral conditions of hosts could make an effect on composition of cellular fatty acids of the spirochetal envelope.

Obtained FAME profiles were also used for cluster analysis. Graphically displayed relatedness between organisms was expressed bu Euclidian distance (ED), which means the distance in multi-dimensional space between two strains when their fatty acid compositions are compared. According to the experience, samples linked at ED 10 or less should represent the same species, generally conclusion about relatedness or similarity of sample runs can be made [21b]. The FAME analysis does not seem to be sufficient for identifying of *B. burgdorferi* s.l. isolates to the species level. Fatty acid

Table 2
Fatty acids and alcohols incidence in analyzed strains

Fatty acids[a]	Frequency in			Frequency total (%)
	Borrelia strains	"*Spironema*" strain	Non-identified strains	
Saturated				
$C_{9:0}$	4/9	–	–	4 (23.5)
$C_{10:0}$	4/9	–	6/7	10 (58.8)
$C_{12:0}$	9/9	1/1	7/7	17 (100)
$C_{13:0}$	–	1/1	6/7	7 (41.2)
$C_{14:0}$	9/9	1/1	7/7	17 (100)
$C_{15:0}$	9/9	1/1	7/7	17 (100)
$C_{16:0}$	9/9	1/1	7/7	17 (100)
$C_{17:0}$	9/9	1/1	6/7	16 (94.1)
$C_{18:0}$	9/9	1/1	7/7	17 (100)
Saturated hydroxy				
$C_{12:0}$ 2OH	–	–	2/7	2 (11.8)
$C_{12:0}$ 3OH	8/9	–	2/7	10 (58.8)
Unsaturated				
$C_{12:1}$ AT11–12	–	–	4/7	4 (23.5)
$C_{13:1}$ AT12–13	–	1/1	7/7	8 (47.1)
$C_{16:1}$ ω9c	8/9	–	–	8 (47.1)
$C_{16:1}$ ω5c	–	–	2/7	
$C_{17:1}$ ω8c	8/9	1/1	2/7	11 (64.7)
$C_{18:3}$ ω6c (6,9,12)	4/9	1/1	2/7	7 (41.2)
$C_{18:1}$ ω9c	9/9	1/1	7/7	17 (100)
$C_{18:1}$ ω7c	6/9	1/1	7/7	14 (82.4)
$C_{18:1}$ ω5c	3/9	–	2/7	5 (29.4)
$C_{20:1}$ ω9c	–	1/1	1/7	2 (11.8)
$C_{20:4}$ ω6,9,12,15c	4/9	1/1	–	5 (29.4)
Methyl branched				
$C_{11:0}$ iso	7/9	–	3/7	10 (58.8)
$C_{14:0}$ iso	–	–	2/7	2 (11.8)
$C_{15:0}$ iso	9/9	–	4/7	13 (76.5)
$C_{15:0}$ anteiso	9/9	1/1	4/7	14 (82.4)
$C_{16:0}$ iso	9/9	1/1	–	10 (58.8)
$C_{17:0}$ iso	9/9	–	–	9 (52.9)
$C_{17:0}$ anteiso	9/9	1/1	1/7	11 (64.7)
Branched-chain hydroxy				
$C_{11:0}$ iso 3OH	3/9	–	–	3 (17.6)
$C_{13:0}$ iso 3OH	5/9	–	3/7	8 (47.1)
Cyclo				
$C_{17:0}$ cyclo	1/9	–	3/7	4 (23.5)
$C_{19:0}$ cyclo ω8c	–	1/1	1/7	2 (11.8)
Alcohols				
$C_{16:0}$ N	3/9	1/1	1/7	5 (29.4)
Others				
Summed feature 1[b]	–	1/1	7/7	8 (47.1)
Summed feature 2[c]	–	–	7/7	7 (41.2)
Summed feature 3[d]	9/9	1/1	7/7	17 (100)
Summed feature 5[e]	9/9	1/1	7/7	17 (100)

–: not detected.
[a] Only fatty acids that were presented in more than one strain at a level above 0.1% are listed in the table.
[b] A group of fatty acids ($C_{13:0}$ 3OH and/or $C_{15:1}$ iI/H) that could not be separated by this method.
[c] $C_{16:1}$ iso I and/or $C_{14:0}$ 3OH.
[d] $C_{15:0}$ iso 2OH and/or $C_{16:1}$ ω7c.
[e] $C_{18:2}$ ω6,9c and/or $C_{18:0}$ anteiso.

Table 3
Cellular fatty acid[a] composition (%) of analyzed strains

Strain	$C_{12:0}$	$C_{13:0}$	$C_{14:0}$	$C_{15:0}$	$C_{16:0}$	$C_{17:0}$	$C_{18:0}$	$C_{13:1}$ AT12–13	$C_{18:1}$ ω9c	$C_{18:1}$ ω7c	$C_{15:0}$ iso	$C_{12:0}$ 2OH	$C_{17:0}$ cyclo	Sum. feature 1[b]	Sum. feature 2[c]	Sum. feature 3[d]	Sum. feature 5[e]	Named peaks (%)
B.burgdorferi s.s. B31[T]	0.39	–	3.34	1.02	40.17	1.10	9.17	–	23.80	1.09	4.27	–	0.19	–	–	1.92	8.14	94.60
B. burgdorferi s.s. BR194	0.30	–	2.71	0.99	42.98	1.26	9.03	–	29.01	0.96	1.90	–	–	–	–	1.65	6.43	97.22
B. garinii 20047[T]	0.44	–	2.63	1.10	39.54	0.83	5.44	–	24.01	–	5.97	–	–	–	–	1.47	6.22	87.65
B. garinii BR14	0.28	–	2.25	0.90	42.69	1.07	6.35	–	32.08	0.85	1.03	–	–	–	–	1.41	7.83	96.74
B. garinii BR92	0.29	–	2.28	0.75	39.73	1.04	6.57	–	34.03	1.00	1.60	–	–	–	–	1.65	8.70	97.64
B. afzelii BR122	0.34	–	2.29	0.85	41.46	1.14	5.21	–	34.35	0.96	0.83	–	–	–	–	1.70	8.69	97.82
B. afzelii BR132	0.31	–	1.99	0.82	39.47	1.19	7.65	–	33.32	0.76	1.00	–	–	–	–	1.50	9.81	97.82
B. afzelii VS461[T]	0.35	–	2.13	0.82	39.31	1.11	10.11	–	33.52	–	0.31	–	–	–	–	1.26	9.44	98.36
B. valaisiana VS116[T]	0.24	–	2.85	1.07	53.48	0.97	6.03	–	27.79	–	0.71	–	–	–	–	1.25	3.83	98.22
"*S. culicis*" BR91	0.22	0.47	42.42	3.98	19.90	0.89	4.69	2.52	9.52	0.95	–	–	–	4.23	–	0.15	7.33	97.44
BR116	12.20	0.31	2.62	0.64	37.52	0.39	2.21	1.60	6.03	11.09	–	–	0.97	2.34	10.57	4.08	3.57	96.14
BR134	13.94	0.75	7.49	0.83	31.87	0.35	3.45	0.89	6.96	5.19	0.84	1.24	4.75	1.20	6.55	5.71	4.91	96.92
BR135	10.27	–	6.24	0.47	35.98	0.52	3.58	0.80	6.70	5.77	–	1.58	4.54	1.60	9.60	7.74	4.61	100.0
BR151	30.61	1.04	5.73	0.56	31.28	0.42	2.56	0.39	9.85	4.82	1.13	–	–	0.78	2.36	1.05	4.16	96.74
BR173	11.58	1.27	9.30	1.23	27.69	–	1.68	4.48	13.69	8.27	1.35	–	–	6.45	2.66	1.81	6.52	97.98
BR208	18.90	0.71	3.38	0.66	40.15	0.72	2.24	2.07	7.37	1.98	–	–	–	3.06	11.24	0.53	5.26	98.27
BR231	23.71	0.77	3.73	0.72	32.93	0.54	2.38	1.49	9.95	1.94	2.32	–	–	2.21	8.08	0.96	5.81	97.54

–: not detected.

[a] The fatty acids shown in this table represent at least 1% of the total fatty acids in two analyzed strains.

[b] Group of fatty acids (13:0 3OH and/or 15:1 iI/H) that could not be separated by this method.

[c] Group of fatty acids (16:1 iso I and/or 14:0 3OH) that could not be separated by this method.

[d] Group of fatty acids (15:0 iso 2OH and/or 16:1 ω7c) that could not be separated by this method.

[e] Group of fatty acids (18:2 ω6,9c and/or 18:0 anteiso) that could not be separated by this method.

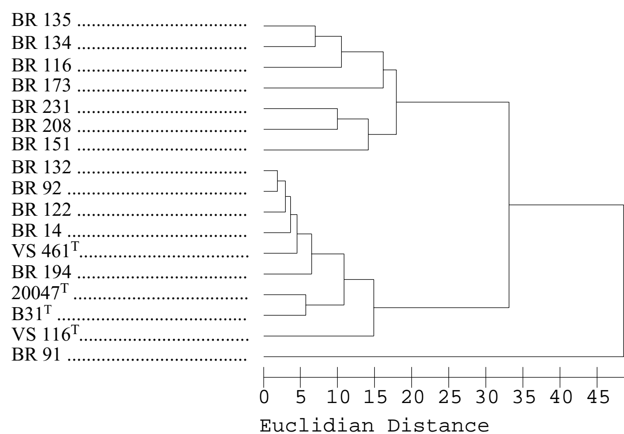

Fig. 1. Dendrogram demonstrating similarity (Euclidian distance) of FAME profiles of all strains tested. BR 135, BR 134, BR 116, BR 173, BR 231, BR 208, BR 151: non-identified spirochetes; BR 132, BR 92, BR 122, BR 14, 20047^{T}: *B. garinii*; VS 461^{T}: *B. afzelii*; BR 194, B 31^{T}: *B. burgdorferi* s.s.; BR 91: "*Spironema culicis*".

composition of included type strains was too close, to show differences between single species. Nevertheless, as shown in differences among major fatty acids, all *B. burgdorferi* s.l. strains cluster separately from the other non-identified spirochetes (Fig. 1). The unclustered strain BR91 proposed as "*S. culicis*" confirms its position as a probably new species.

Fatty acid analysis using gas chromatography in conjunction with MIS system and standard cultivation conditions used is cost-effective method for prime differentiating of spirochetes and should be used as a preliminary characterization of new spirochetal isolates. Easily performed comparison of spirochetal FAME profiles based on cluster analysis could be done prior to more time-consuming methods as ribotyping, DNA–DNA reasociation analysis or plasmid fingerprinting [4]. In conclusion, suggested fatty acid analysis may contribute to the finding of spirochetes in other blood-sucking arthropods or to verify the detection of the same spirochete in different compartment of arthropodal body.

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