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Detection of *Legionella pneumophila* in biofilms containing a complex microbial consortium by gas chromatography–mass spectrometry analysis of genus-specific hydroxy fatty acids

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Abstract: A gas chromatographic–mass spectrometric method was used to detect *Legionella pneumophila* in biofilms in potable water containing a complex microbial consortium. The unique 3-hydroxy and 2,3-dihydroxy fatty acids of the *L. pneumophila* lipopolysaccharides (LPS) were detected in both the planktonic phase of the continuous culture model and in the biofilms forming on both copper and polyethylene substrata. The technique confirmed that lower numbers of *Legionella* colonised and grew on copper in comparison to polyethylene and offers promise for routine detection of *Legionella* in biofilms in the environment.

Key words: Legionella; Biofilms; Continuous culture; Lipopolysaccharide; 3-Hydroxy fatty acids; 2,3-Dihydroxy fatty acids

Introduction

Legionella pneumophila is the major causative agent of Legionnaires disease (legionellosis) and is found ubiquitously in fresh water [1]. The presence of *L. pneumophila* in domestic water circuits has been implicated in several outbreaks of legionellosis [1]. In most cases the surveillance of *Legionella* in environmental water samples is performed by standard microbiological techniques including isolation and quantitation on non-selective and selective agar media, immunofluorescence labelling and hybridisation with nucleic acid probes [2,3].

Biofilms formed on surfaces within water circuits constitute an ecological niche favouring the growth and survival of *Legionella* [4]. In this communication a gas chromatography–mass spectrometry (GC-MS) technique was investigated for the detection of *L. pneumophila* in biofilms formed on plumbing-tube materials. The GC-MS technique is based on measurement of specific *Legionella* fatty acid biomarkers. The lipopolysaccharide (LPS) of *L. pneumophila* contains an exceptionally complex fatty acid composition hitherto not found in any other genera including

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unique 2,3-dihydroxy fatty acids and (v-1)-oxo fatty acids [5,6]. GC-MS measurement of specific LPS derived fatty acids enabled selective detection of *L. pneumophila* in mixed consortia.

Materials and Methods

Bacterial suspensions

A strain of *L. pneumophila* serogroup 1 was used to prepare bacterial suspensions of different concentrations. The concentration of the stock solution was 10^{10} cfu ml^{-1}. Serial dilutions in steps of 10 down to 10^5 cfu ml^{-1} were made. Two 1-ml samples of each concentration were subjected to hydrolysis (see below)

Generation of biofilms

A continuous culture laboratory model was established to generate biofilm samples [7]. The model consisted of five chemostat vessels enclosed within a class III cabinet for the containment of pathogenic bacteria (Fig. 1). Each vessel was composed of glass, titanium and silicone to prevent the ingress of nutrients or extraneous metals which would alter the water chemistry.

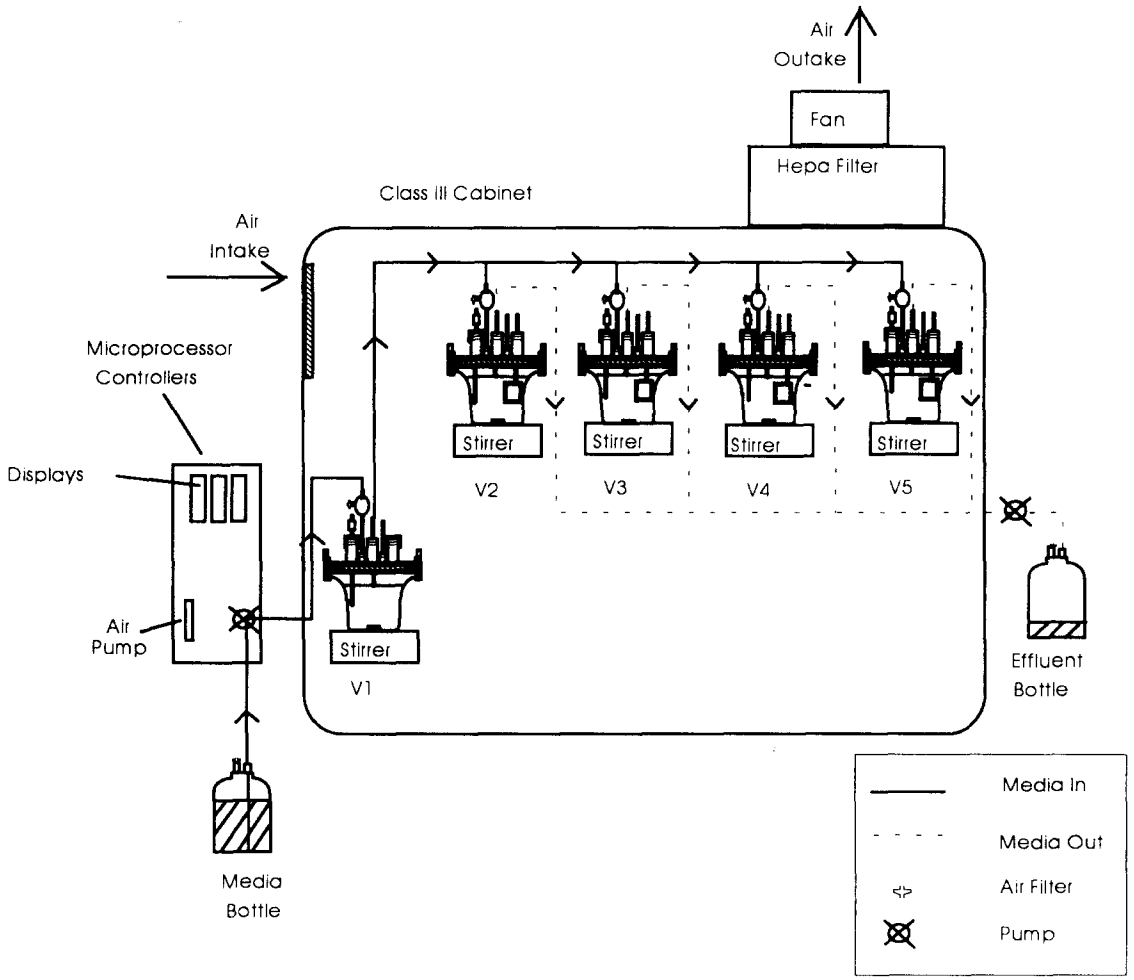

Fig. 1. Schematic diagram of the chemostat apparatus housed within a Class III safety cabinet. V1 denotes the inoculum vessel; V2 contains copper coupons; V3 polybutylene coupons; V4 cPVC coupons and V5 polyethylene coupons.

The inoculum, composed of a mixed consortium of bacteria, was established in the primary vessel at a dilution rate (D) of 0.4 h^{-1}, stirred at 140 rpm and maintained at 30°C. The effluent was used to supply the four chemostat vessels down stream at $D = 0.01$ h^{-1}, 140 rpm and 40°C (to simulate the lower temperatures at which domestic hot water systems operate). When the inoculum had been established (72 h), coupons of different materials (1 cm^2) were suspended in each of the secondary vessels for the generation of biofilms [7]. After 19 days, eight tiles of each material were aseptically removed from each chemostat and sonicated in millipore water for 15 min. Coupons of the following material were studied; copper, polyvinylchloride and polyethylene.

Effluent culture from all vessels was collected over 72 h and filtered through 0.2-μm membranes. The membranes containing the impacted mixed consortia were then vortexed in 10 ml sterile distilled water to resuspend the bacteria.

Sample preparation

Sonicated extracts of the coupons, effluent (10 l) from the chemostats (approx. 6–8 ml) and standard bacterial suspensions were hydrolysed in 4 N hydrochloric acid at 100°C for 4 h whereafter methylesters of fatty acids were as described [8]. Trimethylsilylation (TMS) of hydroxy fatty acids was done with *N,O-bis*(trimethylsilyl)-trifluoroacetamide (BSTFA, Pierce) by adding 1:1 (v/v) BSTFA and methylenechloride:acetonitrile (1:1, v/v) and heating at 85°C for 30 min. After cooling the reaction mixture was injected directly into the GC-MS system.

GC-MS

A Hewlett-Packard GC (model 5890) interfaced to a Hewlett-Packard mass selective detector (model 5971) was used. The GC was equipped with a Restek fused-silica capillary column (30 m × 0.25 mm) coated with Rtx-1 stationary phase. Hydrogen served as carrier gas and the GC temperature was initially 150°C for 2 min, then raised to 300°C at 5°C min^{-1}. Injections were made in the splitless mode with the split valve being opened 30 s after injection. MS analysis was performed in the electron-impact (EI) mode with selected-ion monitoring (SIM) detection using combinations of ions characteristic of the different fatty acid derivatives.

Results and Discussion

Seven LPS-derived fatty acids were chosen as biomarkers for *L. pneumophila*: 3-hydroxyisomyristic acid (*i*14:0(3-OH)), 2,3-dihydroxyisomyristic acid (*i*14:0(2,3-diOH)), 3-hydroxystearic acid (*n*18:0(3-OH)), 3-hydroxyarachidic acid (*n*20:0(3-OH)), 27-oxo-octacosanoic acid (*n*28:0(27-oxo) and heptacosane-1,27-dioic acid (*n*27:0-dioic) [6]. Mass spectra of 3-O-TMS fatty acid methyl esters are characterised by an abundant ion $(M\text{-}15)^+$ [9], which was used for monitoring the 3-hydroxy fatty acids. The TMS derivative of *i*14:0(2,3-diOH) methyl ester forms two structural specific ions m/z 234 and m/z 257 in EI-MS [10]; both ions were used to monitor *i*14:0(2,3-diOH). The methyl ester of *n*28:0(27-oxo) was monitored with ion m/z 362 and *n*27:0-dioic with ion m/z 437, respectively [6]. The SIM profile used for detection of *L. pneumophila* cells is shown in Fig. 2a; the ions obtained with the highest abundance were *i*14:0(3-OH) and *i*14:0(2,3-diOH.

Standard solutions of *L. pneumophila* cells prepared (cell number determined by viable count) were analysed by GC-MS-SIM in order to determine the detection limit of the technique. The detection limit was 10^7 cells. The equation of the standard curve for *L. pneumophila* cells was $y = 1.4x + 260$ ($r^2 = 0.994$) over the range 10^7–10^{10} cfu ml^{-1}.

As several coupons can be used for the same sample and liquid samples can be concentrated by filtration or evaporation prior to hydrolysis, the cell number per coupon or cells per ml can be lower than 10^7.

The polyethylene coupons were found to contain the highest biomass of approximately 10^7 cells of *L. pneumophila* (Fig. 2b), with less detected on the polyvinylchloride coupons and the lowest biomass recovered from the copper coupons. The ratio of the quantitative data obtained by the GC-MS method for hydroxy fatty

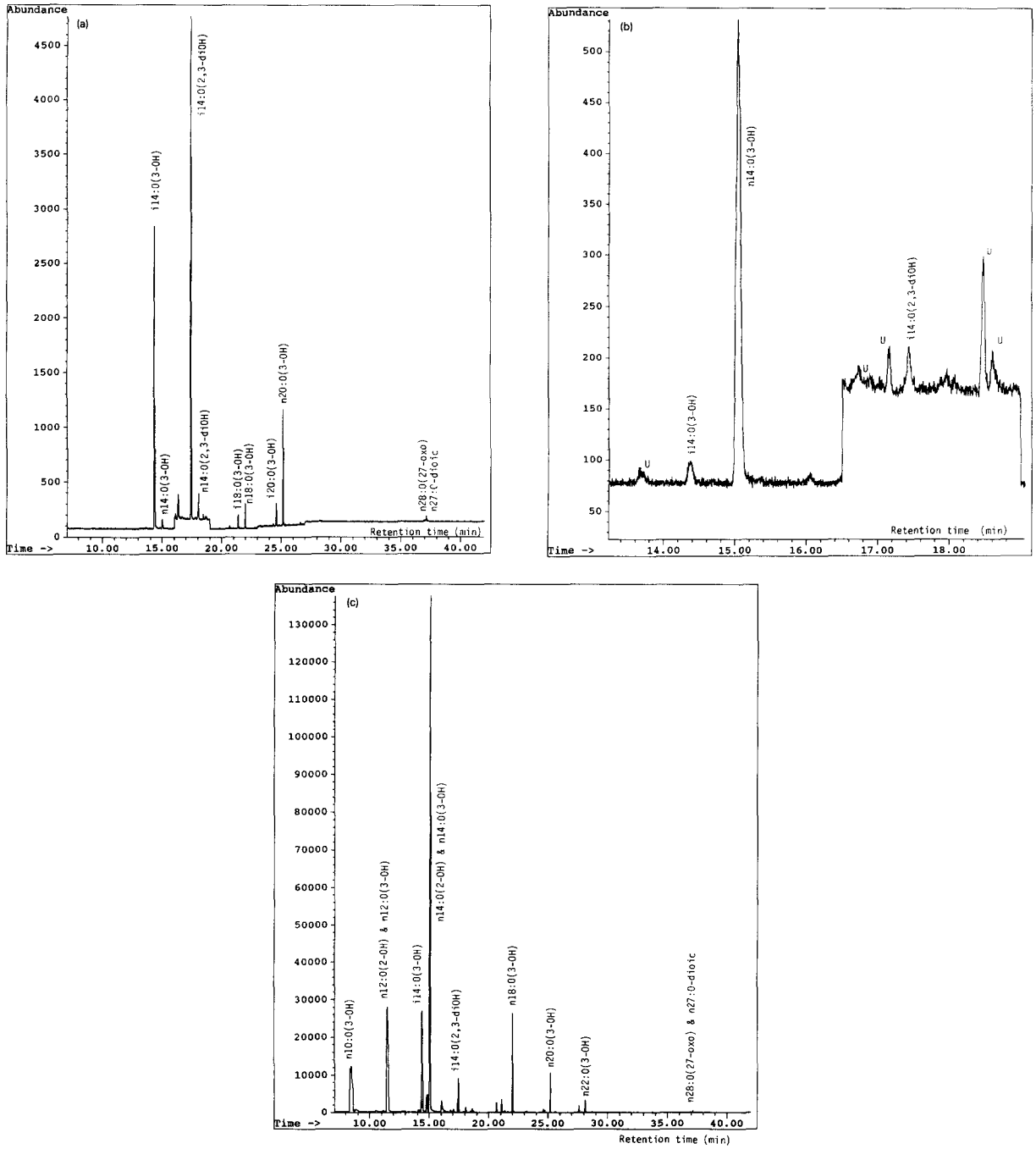

Fig. 2. (**a**) Selected ion current profile of a *L. pneumophila* hydrolysate sample (10^{10} cells) that had been subjected to methanolysis followed by TMS derivatisation. The following ions (at the retention windows indicated) were measured; *m/z* 315 (7–16 min); *m/z* 234 and *m/z* 257 (16–19 min); *m/z* 371 (19–23 min); *m/z* 399 (23–27 min); *m/z* 362 and *m/z* 437 (27–42 min). (**b**) Selected ion current profile of a polyethylene coupon analysed after hydrolysis, methanolysis and trimethylsilylation. SIM at m/z 315 (13–16.5 min) and *m/z* 234 and *m/z* 257 (16.5–19 min) are shown. Unidentified peaks are marked U. (**c**) Selected ion current profile of chemostat effluent after filtration and hydrolysis of the retained solid followed by methanolysis and TMS derivatisation. The following ions (during the retention time windows indicated) were measured: *m/z* 243, *m/z* 259 and *m/z* 287 (7–13 min); *m/z* 271 and *m/z* 315 (13–16.5 min); *m/z* 234 and *m/z* 257 (16.5–19 min); *m/z* 371 (19–24 min); *m/z* 399 (24–26 min); *m/z* 427 (26–32 min); *m/z* 362 and *m/z* 437 (32–42 min).

acid analysis of legionellae on copper and polyethylene substrata (3-fold difference) did not correlate with ratios obtained by viable counts 10^4 *L. pneumophila* and 10^5 from polyethylene surfaces (results not shown), suggesting that there were many dead or dormant legionellae on the copper substratum.

Large amounts (2×10^{10}) of *L. pneumophila* cells were detected in the effluent from the chemostat (Fig. 2c). By the GC-MS-SIM technique the presence of other Gram-negative genera was also detected. High amounts of 3-hydroxycapric (*n*10:0(3-OH)), 3-hydroxylauric acid (*n*12:0(3-OH)) and 2-hydroxylauric acid (*n*12:0(2-OH)) may originate from LPS of *Pseudomonas* species and the high amount of 3-hydroxystearic acid could have originated from ornithine lipids of *Pseudomonas*. 2-Hydroxymyristic acid (*n*14:0(2-OH)) could have originated from sphingolipids of *Sphingomonas* [11] and 3-hydroxymyristic acid (*n*14:0(3-)H)) (higher amounts than *i*14:0(3-OH)), is a common constituent of the LPS of Gram-negative bacteria [12]. These species were also detected by standard microbial techniques (data not shown). A high concentration of *n*14:0(3-OH) was also detected in the coupon samples (Fig. 2b) together with *n*10:0(3-OH), *n*12:0(3-OH), *n*12:0(2-OH) and *n*14:0(2-OH) (data not shown).

Detection of *Legionella* in the environment is difficult due to the poor recovery of viable cells on non-selective and selective agar media and the associated inability to quantitate dead cells. The present study has attempted to utilise unique legionellae biomarkers e.g. 3-hydroxy and 2,3-dihydroxy fatty acids synthesised by *L. pneumophila* in the lipid moieties of their LPS [5]. The data indicate that the methodology is currently capable of detecting 10^7 cells against a high background of complex consortia of microorganisms growing in the planktonic aqueous and the sessile biofilm phases of the environments investigated. GC-MS analysis of electrophilic derivatives of bacterial fatty acids utilising chemical ionization with negative-ion SIM detection has been shown to lower the detection limit of fatty acids by a factor of at least 100 as compared to EI-MS-SIM detection [11]. Thus, by applying similar ionisation techniques to electrophilic derivatives of *Legionella*-specific fatty acids the detection limit could be improved to 10^5 cells or lower.

Of interest, the technique has confirmed that *L. pneumophila* continues to synthesise the 3-hydroxy and 2,3-dihydroxy fatty acids in the planktonic phase and the sessile phases developed on the common plumbing materials, copper and polyethylene. Indeed, the data confirm that low numbers of legionellae colonise biofilms on the copper substratum in comparison to the plastic substratum. The ratios of colonisation shown by GC-MS were a 1:3 ratio on copper and polyethylene, respectively. Whereas the recovery of viable legionellae yielded a 50-fold difference between copper and polyethylene suggesting that a greater proportion of the *L. pneumophila* on copper are non-viable or non-culturable in comparison to polyethylene. Previously [13], classical heat techniques did not appear to revive any potentially dormant legionellae present, which may indicate that the non-recoverable *Legionella* are indeed non-viable. If this is true copper substrata may both reduce colonisation of legionellae and also render them non-culturable.

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