Advantages/Disadvantages/Limitations of Chemotaxis Assays for Campylobacter spp.

Subjects: Biology | Biotechnology & Applied Microbiology

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Chemotaxis directed motility of intestinal bacteria such as *Campylobacter jejuni* could enable the cells to move toward favourable conditions and away from hazardous ones. Reproducible qualitative and quantitative assessment of bacterial chemotactic motility, particularly in response to chemorepellent effectors, is experimentally challenging. Several established chemotaxis assays currently used to investigate *Campylobacter jejuni* chemotaxis are compared, with the aim of improving the correlation between different studies and establishing the best practices.

Keywords: chemotaxis ; Campylobacter jejuni ; chemoeffector screening ; chemotaxis assays

1. Introduction

Chemotaxis directed motility of intestinal bacteria such as *Campylobacter jejuni* enables the cells to move toward favourable conditions and away from hazardous ones and has been shown to be involved in colonisation and disease [1][2][3][4][5][6]. A number of assays have been developed to investigate bacterial chemotaxis [7][8][9], including the capillary and hard plug agar assays (HAP), which are extensively used to study bacterial chemotactic responses to chemoeffectors [10][11]. However, in many cases, the results of different studies lack consistency (particularly when applied to campylobacters) and reproducibility, in addition, they demonstrate excessive experimental variation, unsuitability for studying chemorepellents, and false positive responses [12][13][14][15][16][17]. Moreover, the measurements of migration by chemotaxis assays can be complicated due to the metabolic consumption of chemoeffectors, which may create a secondary gradient that the cells can sense. In order to circumvent these limitations, alternative chemotaxis assays have been developed to investigate the chemotactic behavior of *Campylobacter* spp., including a nutrient-depletion assay, t-HAP assay, tube-based assay, and μ-slide chemotaxis chamber.

2. Agar Plug-Based Assays

Agar plug-based assays were initially introduced for studying chemotaxis of *Escherichia coli* [18][19]. In these assays, a plug of hard agar containing an attractant, or a repellent is placed in a petri dish containing soft agar, at a low enough concentration so that the bacteria can swim, mixed with bacterial cells concentrated enough to be visibly turbid. This assay has been widely adapted and used for other bacteria such as *Shewanella oneidensis, Helicobacter pylori* [20], and *Pseudomonas* spp. [21]. The advantage of this assay is that it is easy to set up, and a response can usually be seen by eye in about 30 min.

2.1. Hard Plug Agar Assay (HAP)

The hard agar plug (HAP) assay, as described by Hugdahl et al. [16], has been extensively used to study changes in campylobacterial chemotactic motility. This is a simple assay where plugs of agar, containing chemoeffectors, are placed in semisolid agar (0.35% agar) containing a dense suspension of bacterial cells (~10^9 cfu/mL). Cells swim in the soft agar through the concentration gradient toward a chemoeffector in the HAP. A visually observable cloudy zone condenses around the HAP if it contains an attractant (positive chemotaxis), or a zone clearing appears around the HAP if contains a repellent (negative chemotaxis). For quantitation, cloudy zones of bacterial cell accumulation around a plug or zones of bacterial clearing, are measured by a ruler from the edge of the plug to the edge of the zone and compared to the control plug. However, the catabolised ligands and their metabolic products could interfere with the accurate measurement of the chemoresponses, as the accumulation of bacterial cells around plugs containing such chemoattractant could create a secondary gradient that the bacteria can sense. For example, catabolised ligand L-serine can be used as a carbon and energy source by *C. jejuni* [22][23]. Serine is converted to pyruvate which is also a chemoattractant for *C. jejuni* [24][25] and induces bacterial growth. In addition, the measurement of the extent of the dense or cleared zones around the HAPs is dependent on the judgement of the operator and can vary from assay to assay and study to study.

While technically undemanding, most HAP-based assays do have a range of limitations and disadvantages, described in **Table 1**, as in both qualitative and quantitative form, these assays rarely produce results in a consistent and reproducible manner [20][26].

Table 1. Advantages and disadvantages of common chemotaxis assays. M- Molar, mM- Millimolar.

Method	Detection Time	Molar Concentration	Advantages	Disadvantages	References
			Agar-based assays		
Hard-plug agar assay (HAP assay)	**3 h**	**10–100 mM**	**-Easy to prepare. -Gives quantitative data. -Requires minimal equipment. -Strains can be compared directly.**	**-Chemorepellent taxis are difficult to observe. -False positive results are possible.**	**[16]**
Modified hard-plug agar assay (t-HAP assay)	**10 min to 3 h**	**10–100 mM**	**-Easy to prepare. -Gives quantitative data. -Requires minimal equipment. -Strains can be compared directly. -Differentiations between catabolised and non-catabolised ligands are possible**	**-Chemorepellent taxis are difficult to observe.**	**[27]**
Nutrient-depletion assay	**3–6 h**	**2–10 mM**	**-Gives quantitative data. -Easy to prepare. -Requires minimal equipment. -Strains can be compared directly. - chemorepellents taxis can be quantitated. -Gradients are created by diffusion, not metabolism.**	**-Sensitive to any motions around the assays. -One strain and conditions can be monitored per assay. -Visual observation is difficult.**	**[28][29]**
Tube-based assay	**75 h**	**1 M**	**-Easy to prepare. -Requires minimal equipment. -Strains can be compared directly.**	**-Not suitable for studying chemorepellents. -Semi-quantitative.**	**[30]**
			Capillary assay		
Capillary assay	**1 h**	**10–100 mM**	**-Gives quantitative data. -Requires minimal equipment. -Gradients are created by diffusion, not metabolism.**	**-Not suitable for studying chemorepellents. -One strain and condition can be monitored per assay.**	**[31]**
			Chemotaxis chamber		
µ-slide chemotaxis chamber	**3 h**	**5–10 mM**	**-Ideal to study the behaviour of a single cell. -Chemoresponses can be measured for a group of cells or a single cell. Clear visualisation of cell migration. -Gives quantitative data.**	**-One strain and condition can be monitored per assay. -Tracking system is relatively expensive.**	**[32][33]**

2.2. Tube-Based Chemotaxis Assays

This assay was first described by Reuter et al. [34] for characterisation of the energy taxis genes, *cj1190c* (*cetA*), *cj1189c* (*cetB*) and *cj1110c* (*cetZ*) in *C. jejuni.* The assay was adapted by Dwivedi, et al. [34] to investigate the fucose chemotaxis in *C. jejuni.* Bacterial cells in 0.4% PBS-agar are transferred to the bottom of a 2 mL Eppendorf tube, allowed to solidify and then overlaid with 1 mL of 0.4% PBS-agar. A filter paper soaked with 50 µL of a chemoeffector (i.e., L-fucose, L-serine) is placed on top of the agar and incubated under microaerobic conditions for 72 h at 37 °C. Bacterial cells that migrate through the upper layer of PBS-agar towards a chemoeffector in the filter paper can be visualised by adding TTC. As TTC changes colour to red in the presence of metabolic activity, the chemoattractant effect can be observed by formation of a red ring of bacterial cells on the top of the tube, visible after 3–4 h of additional incubation [30][35]. The additional advantage of this assay is that the bacteria accumulated in the top layer of the agar can

be collected and quantitated by viable count allowing the collection of both qualitative and quantitative data. Unfortunately, this assay is not suitable for the assessment of chemorepellents and the 72 h incubation time could lead to an increase in cell number due to growth and can thus affect the measurement of chemotactic activity (**Table 1**). The controls became even more difficult to design, as different metabolites affect the increase in the bacterial numbers, due to growth, differently.

2.3. Nutrient-Depletion Assay

The nutrient-depletion assay has been developed for the quantitative assessment of both chemoattractants and chemorepellents [29][36]. Briefly, 0.5% agar (in H_2O without any nutrients) is poured into a petri dish and plugs of 6 mm are removed and then replaced with 0.5% agar with 2 mM of a chemoeffector. The plates are overlaid with 0.1% agar in H_2O and left for 2 h to allow for the diffusion of chemoeffectors to create a chemical gradient. *C. jejuni* cells (~10^8–10^9 cfu/mL) in a 100 µL of bacterial suspension are inoculated in the centre of the petri dish and incubated at 37 °C for 4 h to allow chemotactic migration of the cells. To determine the number of viable bacteria associated with each plug, a 5 mm area around and including each plug is removed and quantitated by viable count. This assay was used to identify ligands for a number of *C. jejuni* chemoreceptors.

3. Capillary Assays

The capillary chemotaxis assay had been considered as a “gold standard” for many years and was the most commonly used method to assess bacterial chemotaxis in which errors due to metabolic activity and growth can be minimized [18][37][38]. The chemotaxis is monitored by measuring the number of bacterial cells entering a capillary tube over a period of hours in the presence or absence of chemoeffectors. In brief, a capillary tube, 1 µL disposable micropipette (3 cm long with an internal diameter of 0.2 mm), containing a solution of an attractant, and sealed at one end, is inserted into a bacterial suspension. A spatial gradient is formed by the diffusion of the attractant/from the tip of the capillary tube. After incubation for 30–60 min, the capillary is removed, and the sealed end is broken off over a test tube containing tryptone broth to be ready for a viable count. For positive chemotaxis, the number of cells accumulated inside a capillary containing attractant solution is measured. For negative chemotaxis, the repellent effector in the capillary decreases the number of cells as opposed to the cell numbers accumulated due to random motion. Driven by the level of handling difficulty, expertise required and low reproducibility, particularly in the assessment of chemorepellents, a number of modifications were introduced over time.

One capillary based assay had been modified to enable the quantitative measurement of bacterial chemoresponses for *Pseudomonas* spp. [39][40][41][42], *H. pylori* [43] and *Campylobacter* spp. [24][31]. Briefly, *C. jejuni* cells are harvested into PBS buffer to OD_{600} of 0.5. A 100 µL of a solution containing 100 mM of a chemoeffector is aspirated through a stainless-steel needle (0.25 mm diameter × 20 mm long) into a 1 mL tuberculin syringe. A 100 µL of the bacterial suspension is then drawn into a 200 µL disposable pipette tip, which is then sealed at one end. The needle-syringe system is fitted to a pipette tip in such a way that most of the needle is immersed into the bacterial suspension and incubated horizontally for 1 h allowing the cells to migrate toward an effector. Bacterial cells migrated into the syringe are enumerated by viable count.

4. Slide-Based Chemotaxis Assay

Recently developed microscopic tracking systems can provide a powerful alternative tool to assess bacterial motility and chemotaxis [44][45][46]. This system allows for a more standardised approach to tracking a group of cells or a single cell through microscopy and time–lapse images measure many features of bacterial motility such as cell migration, velocity, and navigational behavior. A good example is an assay using an agarose-in-plug bridge method, employed to study chemotaxis in many organisms, such as Archaeon *Halobacterium salinarum*, *Escherichia coli*, *P. putida,* and *H. pylori* [21][36][47][48][49]. In principle, two square coverslips are placed on each side of a slide, around 16 mm apart. Agarose plugs are prepared in the middle of the two coverslips by pipetting 5–12 µL of preheated low melting point agarose (LMA), containing the effector to be tested or only PBS as control. Immediately, a third glass coverslip is placed over the bridge, using the edge of the other two coverslips as a stand. The overnight cells are then pipetted between the microscope slide and third glass coverslip and observed by microscopy and photographs are taken of the area at the edges of the plugs after 5–30 min where the chemotactic bands (density of cells) form around the agarose plug. This method is semi-quantitative, aimed at testing attractants and requires skill in assembly of the in-plug bridge. While not used to assess campylobacteria, this method was employed to assess the chemotactic behaviour of *H. salinarum* [47] and demonstrated the cell migration toward glutamate.

5. Comparison of t-HAP, Nutrient-Depletion and µ-Slide Assays

Nutrient-depletion assay, t-HAP and µ-slide chemotaxis appear to offer the most advantages for assessing both chemoattractant and chemorepellent responses. Here, quantitative data is compared from previously published t-HAP, nutrient-depletion and µ-slide assays [27][36] for measurements of the chemotactic motility of *C. jejuni* 11168-O, and its *Δtlp10*^{LBD} isogenic mutant strain [33]. All three assays were in agreement in establishing the repertoire of chemoattractants and chemorepellents for Tlp10.

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