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An improved method for the automated enumeration of fluorescently labelled bacteria in human faeces

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Abstract

The study aimed to improve microscopy-based automated recognition of faecal bacterial cells labelled with 16S rRNA-targeted oligonucleotides and 4′,6-diamidino-2-phenylindole (DAPI). Based on the software KS400 (Carl Zeiss Vision, Hallbergmoos, Germany), designed for automising microscopy-based image capture and image analysis, a routine was developed that affords the recognition of doubly stained bacteria and the rejection of artefacts. The accuracy of the automated enumeration was investigated by comparing the resulting data with those obtained by manual counting. The newly developed method was subsequently used to compare the total bacterial counts in human faecal samples using the domain specific probe Eub338 alone and a mixture of 5 domain-specific probes, respectively. Faecal samples from 90 healthy volunteers were analysed. The cell counts obtained with Eub338 were 10% lower than those obtained with the probe mixture. Since the cells detected with the probe mixture covered a wide range of signal intensities, a dynamic analysis routine was developed to effectively detect the whole range of bright to weak signals within the same image, while at the same time reliably rejecting artefacts.

Keywords: FISH; Image analysis; Automisation

1. Introduction

The microbial community resident in the human gastrointestinal tract has been implicated in a number of health related functions. To investigate the exact role of human intestinal microbiota it is mandatory to know the bacteria that make up this microbial community. The use of culture dependent methods only reveals a small proportion of the species actually present, because a considerable fraction of the bacteria eludes cultivation (Langendijk et al., 1995; Suau et al., 1999). In recent years, molecular methods such as dot blot hybridisation, PCR-based techniques and fluorescence-in-situ-hybridisation (FISH) have been used to enumerate dominant bacterial groups of the human gut microbiota (for reviews see: (Amann et al., 1995; Namsolleck et al., 2004). These methods do not

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require culturing and therefore allow the specific enumeration of bacterial groups or species of interest and the detection of bacteria that do not grow under laboratory conditions.

To analyse large numbers of faecal samples by microscopy-based FISH in a reasonable amount of time, the detection and enumeration of fluorescently labelled cells has to be automated. However, it is also important to make sure that the results obtained by the automated analysis are accurate and reproducible. In recent years, several automated analysis systems for fluorescence microscopy have been described (Bloem et al., 1995; Daims et al., 2001; Schönholzer et al., 2002; Pernthaler et al., 2003). In contrast, the combined application of DNA staining and fluorescently labelled 16S rRNA-targeted oligonucleotide probes has been described in only one study (Schönholzer et al., 2002). However, in this study, which aimed to enumerate bacteria in leaves, cast and the gut of earth worms, the precision of the total cell counts varied in dependence of the total number of bacteria, i.e. higher numbers of bacteria were counted with more precision than lower ones. This was mainly due to the autofluorescent debris particles in the samples which were erroneously counted as bacterial cells. Autofluorescent particles, such as plant residues or mineral particles are also common in faecal samples. To reliably exclude these particles and similar artefacts from being counted, it is necessary to discriminate between autofluorescent particles and fluorescently labelled cells. Here, we describe an automated analysis system, that is more sensitive and approximately 14 times faster than the system described by Schönholzer et al. (2002) and that is able to exclude artefacts from being enumerated.

2. Materials and methods

2.1. Faecal sample preparation

Human faecal samples were collected and processed within 3 h as follows: 0.5 g of faeces was diluted in 4.5 ml PBS buffer (150 mM NaCl, 10 mM Na_2HPO_4, 20 mM NaH_2PO_4, pH 7.4) and homogenised by the addition of 6 glass beads (3 mm in diameter) and vortexing for 3 min. Subsequently, the samples were centrifuged at 300 $\times g$ for 1 min to remove glass beads and larger particles. One milliliter of the suspension was mixed with 3 ml of paraformaldehyde (4%) and incubated for 3 h at 4 °C. Following fixation, 1 ml of the cell suspension was centrifuged at 8000 $\times g$ for 3 min and the cell pellet was resuspended in 300 μl of PBS buffer, mixed with 300 μl of ethanol and stored at −20 °C until use. For the microscopic sample preparation, adhesive, teflon coated 8-well-slides (Roth, Karlsruhe, Germany) were used. The wells had a diameter of 6 mm. The fixed samples were diluted 100-fold and homogenised by shaking them for 1 min at full speed in a Gyroprep (UniEquip Laborgerätebau und-Vertriebs GmbH, Martinsried, Germany). Ten microliters of a detergent solution (0.01% Tween20 in water) was applied to each well, to which 10 μl of the cell suspension was added and mixed by pipetting up and down. The suspension was allowed to air dry and was dehydrated for 3 min each in 60%, 80% and 96% ethanol. Subsequently, the cells were treated with lysozyme (12.5 μl of 0.1% lysozyme in 100 mM TrisHCl pH 8.0 with 50 mM EDTA) for 10 min at 4 °C. The slides were air dried and dehydrated in ethanol as described above. Each well was covered with a mixture of 10 μl of hybridisation buffer (900 mM NaCl, 10 mM TrisHCl pH 7.4, 0.01% SDS) and 2 μl of probe solution (Eub338: 10 pM in hybridisation buffer, EubMix: 2 pM for each probe). The probes used in this study are listed in Table 1. All probes were commercially synthesised (Thermo Hybaid, Ulm, Germany) and 5′-labelled with Cy3 (Mujumdar et al., 1993). The slides were kept in a moist chamber for 3 h at 46 °C and subsequently washed in hybridisation buffer for 30 min at 48 °C. Finally, the slides were immersed in a DAPI solution (200 ng/ml in PBS buffer) for 10 min at room temperature, washed in PBS buffer for 10 min, air dried, mounted with a drop of Vectashield (Vector Labs, Peterborough, UK) and covered with a cover slip. During the hybridisation and afterwards, the samples were protected from light as good as possible to avoid photobleaching.

2.2. Image acquisition

A fully motorised Axioplan2 imaging microscope (Carl Zeiss, Oberkochen, Germany), equipped with a servo-controlled microscope stage (EM14MOT,