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Detection of foodborne bacterial zoonoses by fluorescence *in situ* hybridization

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ABSTRACT

The efficient and timely detection of bacterial pathogens remains a major public health concern throughout the world. Fluorescence *in situ* hybridization (FISH) is a promising tool to detect bacteria since it incorporates the advantages of rapid detection methods with the live/dead differentiation capacity of the gold standard culture methods. However, multiplexing pathogen detection, weak FISH signals and the establishment of a quantitative and sensitive direct enumeration approach remain troublesome obstacles for a widespread use in food microbiology. Therefore, we developed and tested a comprehensive set of highly specific multiplex-FISH tests for the simultaneous detection of various foodborne bacterial zoonoses, including important pathogens like *Salmonella enterica*, thermophilic *Campylobacter* and *Listeria monocytogenes*. The detection of thermophilic *Campylobacter* spp., the most frequent bacterial zoonosis in the EU, in artificially spiked chicken breast by FISH proved to be as sensitive as the conventional ISO standard, but results were available much earlier. Strongly enhanced FISH signals for *Campylobacter* spp., enabling detection in matrices with high background fluorescence, were accomplished by employing several probes for this target group. For the direct detection of bacteria, independent of cultural enrichment, filtration proved to be appropriate although this method is less sensitive and thus primarily suitable for higher bacterial loads. The type of membrane filter as well as the fluorescence channel significantly influenced the efficiency of detection. Furthermore, the implementation of GFP-expressing bacteria as a quantitative standard allowed the enumeration of target pathogens after filtration. In summary, our results demonstrate the applicability of FISH for food microbiology and offer new solutions for prevalent problems in FISH-testing.

1. Introduction

Foodborne illnesses caused by zoonotic bacteria (e.g. thermophilic *Campylobacter* spp., *Salmonella*, *Listeria*, Enterobacteriaceae) pose a serious health hazard in developing countries and remain widespread throughout high-income regions like the European Union (EU) or the United States (US). In the EU more than 200,000 human cases are reported every year (EFSA and ECDC, 2015) with a presumably much higher real incidence. Accordingly, studies in the US calculate with a number of approximately 2 million cases for campylobacteriosis and salmonellosis alone (Scallan et al., 2011). Pathogen-specific cultivation is the standard procedure for bacterial detection (Ge & Meng, 2009). However, the time requirements for these gold standard techniques (e.g. ISO methods) are high and the work load per sample considerable. Rapid methods, most notably immunological and nucleic-acid based methods have gained in popularity, since they are suitable for cheap, high-throughput applications and yield results much faster (Jasson, Jacxsens, Luning, Rajkovic, & Uyttendaele, 2010; Velusamy, Arshak, Korostynska, Oliwa, & Adley, 2010). Unfortunately, these less labour-intensive methods have the decisive disadvantage that they are limited in their ability to distinguish between viable and dead bacteria, especially in the presence of high concentrations of dead bacteria (Birch, Dawson, Cornett, & Keer, 2001; Ge & Meng, 2009; Jasson et al., 2010). Therefore, detection tools which rely on viability markers like ribosomal RNAs, membrane integrity and chemical responsiveness are more suitable to assess the microbial risk posed by food and drinking water. Fluorescence *in situ* hybridization (FISH) is such a promising whole cell detection method,

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which targets 16S or 23S ribosomal RNAs. Originally used to examine the composition of microbial communities in various environments, it has increased in importance for diagnosing human infections as well as for ensuring food safety (Rohde, Hammerl, Appel, Dieckmann, & Al Dahouk, 2015).

Many initial problems of FISH like insufficient specificity or low fluorescence signals could be partially alleviated in the meantime (Wagner, Horn, & Daims, 2003), whereas other obstacles remain: For instance, several pathogens have been targeted in previous studies in single FISH reactions (Almeida, Azevedo, Fernandes, Keevil, & Vieira, 2010; Moreno et al., 2001; Schmid et al., 2003), but multiplex applications in food microbiology, detecting multiple pathogens within one reaction, are scarce, although highly desirable. Furthermore, for some bacterial genera and species several FISH tests are available and the applicability of FISH and its good performance compared to standard techniques have been demonstrated, especially for large bacteria with high ribosome contents and, consequently, strong signals like *Salmonella* or *Escherichia coli*. In contrast, for other pathogenic agents like the much smaller *Campylobacter*, which represents by far the most frequent bacterial zoonosis in the EU and which detection by culture is rather fastidious, efficient FISH-testing in food matrices has not been proven. Also, the use of concentration methods like filtration to lower the limit of detection by FISH is still in its infancy. The same applies to internal standards which allow quantitative measurements of the abundance of a given pathogen. Here, we tackle the four above mentioned challenges by first developing a comprehensive panel of freely combinable FISH tests for different foodborne bacterial pathogens (I). Using thermophilic *Campylobacter* spp. in a contamination model, characterized by a relatively weak FISH signal, we show, secondly, the general potential of FISH-testing in food microbiology and illustrate straightforward ways for signal enhancement (II). Finally, we propose a direct membrane filter-based FISH assay (III) for general screening purposes without the need for enrichment, which also has the potential for pathogen quantification by adding GFP-expressing bacteria (IV).

2. Material and methods

2.1. Strains, cultivation and preparation for FISH-analysis

All 135 strains used in this study are listed in supplementary Table S1. Forty strains of the Enterobacteriaceae family and 30 strains of *Listeria* spp. were aerobically grown in LB medium at 37 °C (except for *Yersinia* strains, which were grown at 28 °C). The 44 strains of *Campylobacter* spp. and *Arcobacter* spp. were grown either at 37 °C or 42 °C on Mueller-Hinton agar or Columbia blood agar under microaerophilic conditions for 48 h. For spiking purposes *Campylobacter jejuni* strains were grown in NZCYM (Sigma-Aldrich) for 24 h. To obtain fixated cells for FISH, gram-negative bacteria were incubated in 4%-formaldehyde for 2 h, washed three times in PBS and stored in 50% ethanol at −20 °C. Gram-positive bacteria (e.g. *Listeria* spp.) were only fixed in ice-cold ethanol by mixing the sample 1:1 with pure ethanol and stored at −20 °C. For specificity testing, the listed non-target strains were pooled and examined as a mixture of 5–10 different strains. If some bacteria in one of these mixtures showed a positive FISH-staining, all strains in this mixture were tested separately. Likewise, to test the sensitivity of a FISH test, each target strain was examined separately.

2.2. Sequencing of 16S and 23S ribosomal genes

To verify the used strain panel and to avoid species misclassification, amplification of the (genomic) target sequences of the strains under study were performed by using the Taq PCR Master Mix Kit (Qiagen, Germany). For 16S sequencing, Bact-0027-F (5′-GTTTGATCCTGGCTCAG-3′) and Uni-1492-R (5′-CGGCTACCTTGTTACGAC-3′) were employed. For 23S sequencing, 23SYers-F (5′-GGTGAGTCGACCCCTAAGGC-3′) and 23SYers-R (5′-TCGGGTGGAGACAGCCTGG-3′) were used for *Yersinia* spp. and *Klebsiella* spp., 23SCamparc-Fw (5′-GGGTAGAGCACTGAATGGGC-3′) and 23SCamparc-Rv (5′-GTCGGGAGGGACTCTTTGTT-3′) were used for *Campylobacter* spp. and *Arcobacter* spp. and 23SSalmFw (5′-CGAATGGGGAAACCCAGTGT-3′) and 23SSalmRv (5′-GCCGAAACAGTGCTCTACCC-3′) were used for *Salmonella* spp. After a denaturation step at 95 °C for 5 min, PCR amplification included 30 cycles (95 °C for 45 s, 55 °C for 45 s, 72 °C for 1.5 min). Sanger sequencing of the amplified and purified products was performed by Eurofins Genomics GmbH (Germany).

2.3. Probe development

Probe design was done by aligning RNA sequences of target and closely related non-target strains retrieved from public databases using ClustalW (Larkin et al., 2007). Potential target sequences were chosen based on this alignment. Specificity and sensitivity of the probe sequences were confirmed using probeCheck, testprobe and blast (Loy et al., 2008; Quast et al., 2013). By using mathFISH (Yilmaz, Parnerkar, & Noguera, 2011), all probes were designed in a way that they can bind efficiently at 52 °C with a formamide concentration of 15% and a NaCl concentration of 0.9 M. It was aimed to reach $\Delta G^{o}_{overall}$-values (the overall free energy change of hybridization; Yilmaz et al., 2011) between −13.0 and −16.0 kcal/mol to ensure high hybridization efficiencies and theoretical formamide melting concentrations of 21–28% (Table S3), while maintaining good specificities (Table S4). If *in vitro* testing at different formamide concentrations revealed insufficient binding at the desired formamide concentrations, the length of the probe was adjusted (accordingly, longer probes to increase binding in the presence of 15% formamide or, vice versa, shorter probes in case of too strong binding affinities). If necessary, unlabelled competitors were used to block the respective target sites of non-target organisms. If mathFISH analysis indicated a low discriminatory power with the use of DNA competitors, the influence of single mismatches of the competitors and the probes was further strengthened by replacing these nucleotides with the DNA analogs locked nucleic acids (LNAs) (Kubota, Ohashi, Imachi, & Harada, 2006). LNAs were placed in a way that hairpin structures and strong self-complementarity were avoided. Helper probes to break up potential secondary rRNA structures were placed adjacently to the probes in a distance of 3 nucleotides. All FISH probes and oligonucleotides were synthesized by TIB molbiol (Germany) and labelled with Alexa488 (green channel), Texas Red (red channel) or AMCA (blue channel). Probes, competitors and helper probes were delivered as lyophilisates and diluted in distilled water prior to storage at −20 °C.

2.4. FISH

Ten µl of a fixed sample were spread on coated glass slides and dried on a 52 °C hot plate (miacom® diagnostics, Germany). If gram-positive bacteria (for instance *Listeria* spp.) were targeted, the bacteria were permeabilized with 10 µl of lysozyme solution (Carl Roth, Germany, 10 mg/ml) for 5 min at room temperature and afterwards rinsed shortly twice with distilled water. Samples were then dehydrated in 50%, 80% and 96% ethanol for 3–5 min each. The slides were coated with 10 µl hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl (pH 7.2), 0.01% SDS, 15% formamide) containing the probes and competitors as listed in Table 1 and Table S2, respectively. Probe concentrations were set at 500 nM, total