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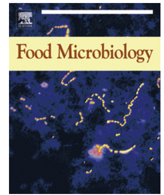

Short communication

Differential detection of pathogenic *Yersinia* spp. by fluorescence *in situ* hybridization

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ABSTRACT

Yersinia enterocolitica, *Y. pseudotuberculosis* and *Y. pestis* are pathogens of major medical importance, which are responsible for a considerable number of infections every year. The detection of these species still relies on cultural methods, which are slow, labour intensive and often hampered by the presence of high amounts of accompanying flora. In this study, fluorescence *in situ* hybridization (FISH) was used to develop a fast, sensitive and reliable alternative to detect viable bacteria in food. For this purpose, highly specific probes targeting the 16S and 23S ribosomal RNA were employed to differentially detect each of the three species. In order to enable the differentiation of single nucleotide polymorphisms (SNPs), suitable competitor oligonucleotides and locked nucleic acids (LNAs) were used. Starved cells still showed a strong signal and a direct viable count (DVC) approach combined with FISH optimized live/dead discrimination. Sensitivity of the FISH test was high and even a single cell per gram of spiked minced pork meat could be detected within a day, demonstrating the applicability to identify foodborne hazards at an early stage. In conclusion, the established FISH tests proved to be promising tools to compensate existing drawbacks of the conventional cultural detection of these important zoonotic agents.

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1. Introduction

Yersinia forms a clinically important genus in the family of Enterobacteriaceae, with three species of human pathogenic relevance, the foodborne pathogens *Y. enterocolitica*, *Y. pseudotuberculosis* and its close relative *Y. pestis*, the causative agent of plague. In the European Union, *Y. enterocolitica* is the third most frequent bacterial zoonosis of foodborne origin after salmonellosis and campylobacteriosis (EFSA and ECDC, 2015). In the USA, there are an estimated 100,000 cases every year; however, according to the Centers for Disease Control and Prevention (CDC) only 1 in 123 cases is diagnosed (Scallan et al., 2011). The detection of *Yersinia* in food is still primarily performed by cultivation. In contrast to other Enterobacteriaceae, the generation time of *Yersinia* spp. is rather long, interfering with fast detection and often resulting in false-negative results due to the overgrowth by accompanying flora (Gupta et al., 2015). A great diversity of different isolation procedures for *Yersinia* spp. exists, resulting in considerable variations in the isolation success (Fukushima et al., 2011). Rapid detection methods, such as PCR, have been, therefore, employed to allow a more efficient screening of food products (Gui and Patel, 2011; Gupta et al., 2015). However, most molecular methods cannot distinguish between living and dead bacteria and some selective enrichment media contain PCR inhibitors which have to be removed beforehand (Lantz et al., 1998). Fluorescence *in situ* hybridization (FISH), which targets the more fragile ribosomal RNAs and stains whole cells, is a promising alternative approach in food microbiology (Rohde et al., 2015). Better design tools as well as the use of nucleotide analogues like peptide nucleic acids (PNAs) and locked nucleic acids (LNAs) have facilitated the implementation of FISH tests (Cerqueira et al., 2008; Noguera et al., 2014; Yilmaz et al., 2011). FISH can also be combined with other tests for live/dead differentiation, most notably the direct viable count method (DVC) (Moreno et al., 2012; Piqueres et al., 2006; Wu et al., 2009). In the DVC assay, bacteria are confronted with sublethal concentrations of an antibiotic substance, which inhibits cell division, but not cell

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growth, resulting in elongated cells, whereas dead cells remain unchanged (Kogure et al., 1979). Here we describe a comprehensive FISH test, able to differentially detect *Y. enterocolitica*, *Y. pseudotuberculosis* and *Y. pestis*. We established a set of specific probes and competitors, examined the effects of bacterial long-term storage on the FISH signal and propose a combined DVC-FISH assay to prevent false-positive detection of dead cells. To assess its suitability for the application in food products, we compared the performance of FISH with the standard detection procedures of ISO 10273:2003 in artificially spiked minced pork meat.

2. Materials and methods

2.1. Strains and cultivation

All *Yersinia* strains were cultivated in lysogeny broth (LB) under aerobic conditions at 28 °C (Table S1). Other non-target strains (Table S1) were cultivated aerobically (e.g. other *Enterobacteriaceae*) or anaerobically (e.g. *Campylobacteraceae*) at 37 °C under rotational shaking (180–220 rpm/min).

2.2. Sample fixation, fluorescence in situ hybridization (FISH) and microscopy

Fixation was performed following a standard fixation procedure (Amann et al., 1990) by centrifugation at 14,000 *g* and resuspension of the pellet in 4% PBS/formaldehyde mixture (Carl Roth, Germany), incubation for 2 h at 4 °C and washing three times with PBS. Finally, cell pellets were resuspended in a 50% ethanol/PBS mixture and stored at −20 °C. Alternatively, cultures of *Y. enterocolitica* or *Y. pseudotuberculosis* were quickly fixed in one step by the addition of one volume of icecold 100% ethanol, pre-cooled to −20 °C, and stored at −20 °C.

For FISH-analysis, 10 µl of the sample was placed on coated glass slides, dried completely on a 52 °C hot plate (miacom® diagnostics, Germany) and dehydrated in successive steps in 50%, 80% and 96% ethanol for 3–5 min each. Slides were coated with 10 µl hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl (pH 7.2), 0.01% SDS, 15% formamide; 50% formamide for Yerspestis1523 and Yerspseu1523) containing the probes and competitors as listed in Table 1 and Table S2. Probe concentrations were set at 500 nM, total corresponding competitor concentrations were set at 250 nM (equimolar amounts for multiple competitors). Hybridization was performed in a light-protected humidity chamber at 52 °C for 1 h. Slides were then carefully immersed in distilled cold water for a few seconds, followed by washing for 10 min (310 mM NaCl, 20 mM Tris-HCl (pH 7.2), 0.01% SDS) at 52 °C (no low-salt washing steps for Yerspseu1523 and Yerspest1523). Slides were again immersed in water, rapidly air-dried and embedded in Roti®-Mount FluorCare DAPI (Carl Roth, Germany). For each hybridization reaction, a set of controls was carried out in parallel: Pure cultures of the target strain (e.g. *Y. enterocolitica* DSM 13030 or *Y. pseudotuberculosis* ATCC 29833) and closely related non-target species (e.g. non-pathogenic *Yersinia* spp. like *Y. intermedia* ATCC 29909) served as positive and negative controls. Matrix controls with and without *Yersinia* were used to evaluate the signal-to-noise ratio and to estimate the amount of autofluorescent particles.

Microscopic evaluation was performed with an AxioScope fluorescence microscope by employing a 100x N-achroplan Ph3 M27 oil objective (Zeiss, Germany). Images were acquired by the AxioCam MRm and further processed for overlay of different fluorophore channels by using the imaging software ZEN 2012 (Zeiss). Every positive FISH signal in one channel was only considered as true-positive, if the confirmatory probe in the other channel (e.g. YersEcoI16-Alexa488/YersEcoII16-Alexa488 in combination with the confirmatory probe YersEco23-TexasRed) and the genus-specific probe Yersall-Demaneche showed comparable hybridization signals (Table 1).

To compare the fluorescence intensities of different samples, exposure times and LED output were fixed at a level where the fluorescence of the sample with the brightest signal intensity was close to detector saturation. In each overlay image 50 cells were quantified by using the ZEN 2012 intensity measurement. Background correction was done for each image separately by measuring the intensity of a large cell-free region and subtracting this value from the obtained FISH signals. All data are given as means with standard deviations.

2.3. Sequencing of 16S and 23S ribosomal RNA

The genomic target sequences of the different *Yersinia* strains were amplified by using the Taq PCR Master Mix Kit (Qiagen, Germany). For 16S and 23S sequencing, Bact-0027-F (5′-GTTTGATCCTGGCTCAG-3′) and Uni-1492-R (5′-CGGCTACCTTGTTACGAC-3′) and 23SYers-F (5′-GGTGAGTCGACCCCTAAGGC-3′) and 23SYers-R (5′-TCGGGTGGAGACAGCCTGG-3′) were used, respectively. After an initial 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 sequences was performed by Eurofins Genomics GmbH (Germany).

2.4. Probe development and testing

Probe design and testing was carried out as previously described (Rohde et al., 2016). Briefly, probe sequences for *Y. enterocolitica*, *Y. pseudotuberculosis* and *Y. pestis* were deduced by aligning RNA sequences of target and closely related non-target species (e.g. other *Yersinia* spp.) using ClustalW (Larkin et al., 2007). Specificity and sensitivity of potential probe sequences were confirmed using probecheck, testprobe and blast (Loy et al., 2008; Quast et al., 2013). If necessary (e.g. less than two mismatches between target and

Table 1
Probes used in this study.

Probe name	Sequence (5′–3′)	Target	Purpose
YersEcoI16	TATTAAGTTATTGGCCTTCCTCCT	16S	Detection of *Y. enterocolitica*
YersEcoII16	TTAACCTTTATGCCTTCCTCCTC	16S	Detection of *Y. enterocolitica*
YersEco23	CAAGTCCCTTTACCTAATGCCAGC	23S	Confirmation of *Y. enterocolitica*
YersPseu23	ATCACGCCTCAGGGTTGATAAG	23S	Detection of *Y. pestis* and *Y. pseudotuberculosis*
YersPseu16	GCGTATTAAACTCAACCCCTTCC	16S	Confirmation of *Y. pestis* and *Y. pseudotuberculosis*
YersPest1523-TexasRed	CTGCACCGTGGTGCATCGTC	23S	Detection of *Y. pestis*
YersPseu1523-Alexa488	CTGCACCGTAGTGCATCGTC	23S	Negative confirmation of *Y. pestis*
Yersall-Demaneche	GTTCGCTTCACTTTGTATCT	16S	Detection (confirmation) of *Yersinia* spp.
EUB-338	GCTGCCTCCCGTAGGAGT	16S	Detection of all bacteria

Underlined nucleotides represent LNAs.

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