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Multiplex primer-extension assay for identification of Yersinia species

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Abstract A multiplex primer-extension reaction (PER) assay, was specifically designed for the identification of ten *Yersinia* species. The assay, directed towards the *tuf*A (elongation factor Tu) gene, was tested on a total of 42 samples representing *Yersinia* species and non-*Yersinia* species. The primers used in the preliminary PCR, designed in highly conserved regions upstream and downstream of the diagnosis sites, successfully amplified a 587 bp fragment. The diagnosis sites were simultaneously interrogated using a multiplex PER and the results were confirmed by fragment sequencing. The proposed test provides an appropriate tool to monitor the presence of *Yersinia* spp. in food samples and to evaluate the potential hazard for consumers.

Keywords Multiplex primer-extension reaction · Species identification · *tuf*A · *Yersinia* spp.

The genus *Yersinia* belongs to the family *Enterobacteriaceae* and comprises 18 species (http://www.bacterio.net/xz/yersinia.html). Among these, some species like *Yersinia pseudotuberculosis,* and *Yersinia enterocolitica* are enteropathogens that cause foodborne illness. While most of the attention is focused in these species, several other less familiar *Yersinia* species (*Yersinia aldovae, Yersinia bercovieri, Yersinia frederiksenii, Yersinia intermedia, Yersinia kristensenii, Yersinia mollaretii, Yersinia rohdei* and *Yersinia ruckeri)*, isolated from food and environment, are considered opportunistic pathogens [1].

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Conventional methods for the identification of *Yersinia* spp., such as a biochemical profiling and serological tests, are time-consuming and limited by their poor ability to differentiate strains within some species and sometimes by the low reproducibility [2].

Recently, many genotypic methods have been developed for the identification of human pathogenic species like *Yersinia pestis, Y. pseudotuberculosis* and *Y. enterocolitica* [3].

Furthermore rapid, specific and multitarget methods focusing on less studied species are needed [4].

Minisequencinq is a novel technology for quickly detecting and identifying multiple Single Nucleotide Polymorphisms (SNPs). In particular, this technique is based on the simultaneous analysis of several SNPs by means of multiplex primer-extension reaction (PER) in conjunction with a genetic analyser [5].

The aim of the study was the development of a minisequencing test to differentiate several *Yersinia* species frequently isolated from food samples, including both human pathogens and opportunistic pathogens (*Y. aldovae, Y. bercovieri, Y. enterocolitica, Y. frederiksenii, Y. intermedia, Y. kristensenii, Y. mollaretii, Y. pseudotuberculosis, Y. rohdei* and *Y. ruckeri*).

All reference strains used to develop the assay are listed in Table 1. Bacterial strains belonging to phylogenetically related genera were also tested to verify possible cross-reactions (Table 1).

Moreover in order to evaluate the possible applicability of the proposed assay, 18 wild isolates (WI) obtained from meats of large game animal hunted in Italian Alps were used (six *Y. rohdei,* six *Y. kristensenii,* one *Y. enterocolitica*, four *Y. intermedia,* one *Y. aldovae*) (Table 1).

All the isolates were grown overnight at 31 °C in tryptone soya broth (TSB, Acumedia). DNA was extracted by

Table 1 Bacterial strains used to develop the multiplex PER assay

Species	Source	Strain
Reference *Yersinia* strains		
Yersinia aldovae	CIP	103162
Yersinia bercovieri	CIP	103323
Yersinia enterocolitica	CIP	65.29
Yersinia enterocolitica	CIP	72.5
Yersinia enterocolitica	CIP	80.27
Yersinia enterocolitica	CIP	81.42
Yersinia enterocolitica	CIP	101776
Yersinia enterocolitica	CIP	106676
Yersinia enterocolitica	CIP	107202
Yersinia enterocolitica	CIP	106945
Yersinia frederiksenii	CIP	80.29
Yersinia frederiksenii	CCUG	26949
Yersinia frederiksenii	CCUG	82.46
Yersinia intermedia	CIP	80.28
Yersinia kristensenii	CIP	80.30
Yersinia massiliensis	CIP	109351
Yersinia pseudotuberculosis	CIP	A1
Yersinia rohdei	CIP	103163
Yersinia ruckeri	CIP	82.80
Negative control strains		
Escherichia coli	ATCC	25922
Proteus vulgaris	ATCC	29513
Salmonella enterica subsp. *enterica*	ATCC	10708
Citrobacter freundii	ATCC	13316
Enterobacter sakazakii	ATCC	BAA-894
Wild isolates[a]		
Y. rohdei	–	1–6
Y. kristensenii	–	1–6
Y. enterocolitica	–	1
Y. intermedia	–	1–4
Y. aldovae	–	1

CIP Collection de l'Institut Pasteur, *CCUG* Culture Collection University of Göteborg, *ATCC* American Type Culture Collection

[a] For the isolation of WI, *Yersinia* spp. was detected using a previously published protocol [10]. Successively, the WI isolated colonies were identified by means of the MicroSeq 500 16S rDNA bacterial sequencing kit (Applied Biosystems)

boiling procedure and quantified using a spectrophotometer (Nanodrop 2000, Thermo Fisher Scientific).

The *tuf*A (elongation factor Tu) sequences obtained from the GenBank database (National Center for Biotechnology Information, NCBI, http://www.ncbi.nlm.nih.gov), corresponding to *Y. aldovae* (accession no. EF113987), *Y. bercovieri* (accession no. EF113993), *Y. enterocolitica* (accession no. EU566885), *Y. frederiksenii* (accession no. EF114009), *Y. intermedia* (accession no. EF114012), *Y. kristensenii* (accession no. EF114016), *Y. mollaretii* (accession no. EF114019), *Y. pseudotuberculosis* (accession no. EU566891), *Y. rohdei* (accession no. EF114027) and *Y. ruckeri* (accession no. EF114030) and other species belonging to genera correlated, were aligned with the ClustalV program [6] for the detection of SNPs to be used as diagnosis positions.

The *tuf*A gene was selected because it has been proven to be valuable for accurate evaluation of genetic relationships among closely related microorganism such as the members of the family *Enterobacteriaceae*. In particular within the *Yersinia* genus, the *tuf*A gene has a remarkable level of interspecies variability (16 %) [7].

Analysis of the alignment of the reference sequences showed that eight SNPs at positions 72, 93, 130, 171, 417, 474, 501, and 531 (numbering referred to GenBank accession no. EU566891), could differentiate the *Yersinia* species most commonly isolated from food and environment, providing specific minisequencing profiles (Table 2).

All the sequences available for each *Yersinia* species in GenBank were examined to confirm the absence of intraspecific variations of the diagnosis sites that have been selected.

Primers for the preliminary PCR have been designed upstream and downstream the diagnosis sites in well-conserved regions within the *Yersinia* genus (Table 3). Sequencing primers (SP) were designed immediately flanking the diagnostic sites and had varying lengths of poly(dT) non-homologous tails attached to the 5′ end (Table 3).

Preliminary PCR reactions were performed in 50 μL volumes consisting of 20 mM Tris–HCl, 1 U of recombinant *Taq* DNA polymerase (Invitrogen), 0.2 mM each of dNTPs (Invitrogen), 2 mM $MgCl_2$, 25 pmol of each primer and 50 ng of DNA. PCR conditions were 3 min at 94 °C followed by 35 cycles at 94 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min and a final extension at 72 °C for 5 min.

Preliminary PCR allowed the amplification of a 587 bp fragment in all reference *Yersinia* samples. No cross-reaction was detected when the primers were tested with other genera phylogenetically related to *Yersinia* genus.

The obtained PCR products acted as templates for the PER reaction after the enzymatic clean-up with Exo-Sap (USB Europe GmbH).

Tailed sequencing primers were tested individually to assess their performance and validate migration size before the eight primers were tested in multiplex reaction.

The multiplex PER was performed according to the SNaPshot multiplex kit protocol (Applied Biosystems, Foster City, CA) and using the following concentrations of sequencing primers: 0.12 μM of SP1, 0.18 μM of SP2, SP5 and SP6; 0.24 μM of SP8; 0.30 μM of SP3 and SP4; 0.48 μM of SP7.

Table 2 Minisequencing profiles on the basis of the alignment of the reference sequences

	SP1rev (93)	SP2 (531)	SP3rev(417)	Sp4 (171)	SP5 (474)	SP6rev(501)	SP7rev(72)	SP8rev(130)
Y. aldovae (EF113987)	T	C	G	T	C	T	A	C
Y. bercovieri (EF113993)	C	C	G	T	C	T	A	T
Y. enterocolitica (EU566885)	C	C	A	T	C	T	G	C
Y. frederiksenii (EF114009)	C	C	G	T	C	C	A	T
Y. intermedia (EF114012)	C	C	G	T	C	T	A	C
Y. kristensenii (EF114016)	C	C	G	T	C	T	G	C
Y. mollaretii (EF114019)	C	C	G	T	T	T	A	C
Y. pseudotuberculosis (EU566891)	C	C	A	C	C	T	G	C
Y. rohdei (EF114027)	C	C	G	T	C	T	G	T
Y. ruckeri (EF114030)	C	T	G	T	C	T	G	C

The numbers in parenthesis indicates the position number of the diagnosis sites interrogated by the sequencing primers (as referred to *Y. pseudotuberculosis tuf*A gene sequence, GenBank accession no. EU566891)

Table 3 Primers for preliminary PCR and PER

	Primers	Primer position	Primer sequence	Diagnosis position interrogated[a]
Preliminary PCR[b]	Sense	41–59	5′-ACATCCTGTTGGGTCGYCA-3′	–
	Antisene	628–603	5′-TCTTTGCTCAGAATATAAACTTCTGA-3′	–
PER[c]	SP1rev	113–94	5′-TCATCAACCATGTCASATTT-3′	93
	SP2	516–530	5′-11(T)TCTGYTGCGTGGTAT-3′	531
	SP3rev	439–418	5′-9(T)CAGTATCTTTGATACCRACGAT-3′	417
	SP4	153–170	5′-19(T)ACTTCTTTCTGCTTACGA-3′	171
	SP5	457–473	5′-26(T)GGCGTTGAAATGTTCCG-3′	474
	SP6rev	518–502	5′-33(T)AGAACACCAACGTTCTC-3′	501
	SP7rev	96–73	5′-35(T)GTTCATGAATACGATSATGTA-3′	72
	SP8rev	148–131	5′-44(T)GAACTTCCATTTCTACCA-3′	130

[a] Diagnosis positions are referred to *Y. pseudotuberculosis tuf*A gene sequence (GenBank accession no. EU566891)

[b] Primers for the preliminary PCR were designed upstream and downstream from the diagnostic SNPs

[c] SPs were designed immediately flanking the diagnostic sites and with varying lengths of nonhomologous poly(dT) tails attached to their 5′ ends

PER products were cleaned using 1 unit of calf intestine alkaline phosphatase (CIAP) (Fermentas) at 37 °C for 1 h and at 75 °C for 15 min.

Finally, samples were prepared by adding 1 μL of the post-PER product to 24.6 μL of formamide (Applied Biosystems) and 0.4 μL of GeneScan 120 LIZ size standard (Applied Biosystems). Each sample was loaded on the ABI 310 Genetic Analyser (Applied Biosystems). Electropherograms were analyzed using the GeneScan 4.0 software (Applied Biosystems).

To confirm multiplex PER results, amplification products of *tuf*A gene were sequenced using ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit, version 1.1 (Applied Biosystems).

All of the *Yersinia* reference strains and WI gave rise to the expected species-specific patterns thus confirming the specificity and applicability of the multiplex PER technique (Fig. 1).

In conclusion, the multiplex PER assay is as precise and reliable as the sequencing test and it has the advantage of being quicker allowing an immediate interpretation of the results [8]. In fact, the discrimination of the 10 species is possible by means of a single read of the generated pattern.

Furthermore, since the minisequencing developed test is a multiplex format assay, it would increase the analytical productivity by reducing analysis costs and reagent consumption [9].

For all these reasons, the test proposed in this paper, applied for the specific detection of *Yersinia* species most frequently isolated from food, represents the ideal solution for routine analysis in laboratories equipped with genetic analyzer. Moreover the developed test can be an important

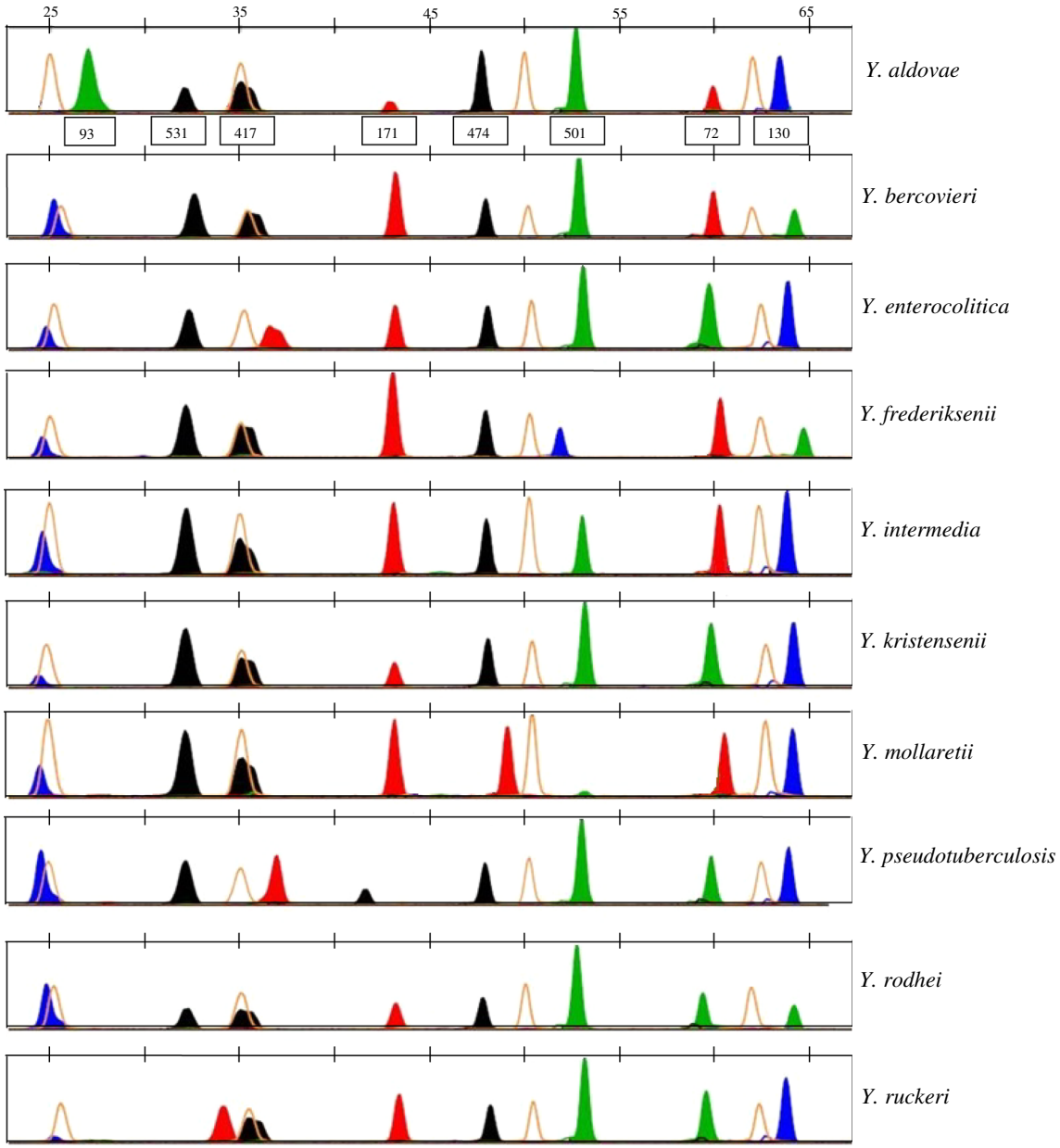

Fig. 1 Species specific patterns observed in reference strains. In the *boxes* are indicated the positions of diagnosis sites interrogated by each sequencing primers (as referred to GenBank accession no. EU566891)

tool for monitoring the ecology of *Yersinia* spp. and as consequence improving the food quality.

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Conflict of interest None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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