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A novel real-time PCR assay for the detection of *Helicobacter pullorum*-like organisms in chicken products

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Summary. A novel real-time PCR assay was developed for the direct detection in food of *Helicobacter pullorum*-like bacteria, which are occasionally associated with human enteric disease. Experiments using control strains showed that the real-time PCR assay was specific and reproducible, with a detection level of 1 colony-forming unit (CFU)/g. The assay was then applied to determine contamination rates in 30 samples of three types of chicken-meat products obtained from five retail outlets in Spain (Valencia); all of the samples were initially considered to be negative for *Helicobacter* even after an enrichment period. *H.-pullorum*-like DNA was detected in seven out of ten chicken carcasses and in one chicken-burger sample (without enrichment), as well as in one liver sample (after enrichment). Sequencing of three randomly selected PCR products confirmed concordance (99% homology) with the *H. pullorum* 16S rDNA gene. The advantages of real-time PCR over conventional PCR assays are the improved detection level, speed of testing, and validation of specificity by melting-point analysis. The fact that bacteria are frequently present in chicken carcasses sold in retail stores highlights the importance of more widely monitoring contamination rates. The novel assay described herein allows better assessment of potential human health risks posed by *H. pullorum*. [**Int Microbiol** 2008; 11(3):XXX-XXX]

Key words: *Helicobacter pullorum* · *Campylobacter* spp. · chicken products · real-time PCR

Introduction

Chicken flocks are frequently colonized by *Campylobacter jejuni* and *Campylobacter coli* [7] and a significant proportion of chicken products sold at retail are contaminated, thus posing a major source of campylobacteriosis in humans [19]. *Helicobacter pullorum*, a related bacterium, is another common inhabitant of the ceca and large intestine of asymptomatic broiler chickens [3]. The bacterium also has been found in the livers and intestinal contents of laying hens with vibrionic hepatitis [22]. *H. pullorum* may also contribute to human disease, as suggested by reports of its isolation from feces and biopsies of patients with gastroenteritis, chronic liver disease, and inflammatory bowel disease [4,22–24]. Avian species also serve as reservoirs for the closely related *Helicobacter canadensis* [2, 11,25], which likewise has been isolated from humans although its role as an enteropathogen remains unclear [11]. The host diversity of helicobacters resembling *H. pullorum* was recently extended by the isolation of atypical genetically distinct strains from swine feces [15]. In addition, the true prevalence of *H. pullorum* and

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related bacteria in poultry and in human and animal disease may be underestimated due to misidentification, since this species shares many phenotypic similarities with *Campylobacter* species and accurate tests to distinguish among them are lacking [2,3]. The fastidious growth requirements of *H. pullorum* necessitate the use of enrichment techniques and special culture medium formulations for selective recovery and detection of the bacterium in foods. These have been adapted from methods developed originally for the isolation of *Campylobacter* [3,6,18], are generally difficult to use, and may have limited specificity. As a consequence, data on the prevalence of *H. pullorum* in retail food, in particular raw chicken, are limited.

The objectives of this study were to develop a rapid and specific real-time PCR assay able to detect *H.-pullorum*-like bacteria in food. The assay was then applied in parallel with direct culture (with and without an enrichment stage) and a previously described conventional PCR assay to determine contamination rates in samples of raw chicken products sold in retail outlets.

Material and methods

Bacterial strains and culture conditions. *Helicobacter pullorum* NCTC 13153 (National Collection of Type Cultures, London, UK) was used as the species reference strain. To validate the specificity of the PCR assays, additional reference strains were used: *Campylobacter coli* NCTC 11366, *C. jejuni* NCTC 11828, *C. lari* NCTC 11352, *Arcobacter butzleri* NCTC 12481, *A. cryaerophilus* NCTC 11885, *A. skirrowii* NCTC 12713, *Salmonella enterica* NCTC 12117, *Enterobacter faecalis* DSMZ 20478 (Deutsche Sammlung von Mikroorganismen und Zellen, Braunschweig, Germany), *E. cloacae* CECT 194 (Colección Española de Cultivos Tipo, Valencia, Spain), *Staphylococcus aureus* CECT 240, *S. epidermidis* CECT 231, *Escherichia coli* CECT 349, *Citrobacter freundii* CECT 401, *Micrococcus luteus* CECT 245, and *Listeria monocytogenes* ATCC 19113 (American Type Culture Collection, USA). *Helicobacter* and *Campylobacter* reference strains were grown on Columbia agar base supplemented with 10% (v/v) defibrinated horse blood (Oxoid, Basingstoke, UK) for 48–72 h at 37°C under microaerophilic conditions (5% H_2, 5% CO_2, 5% O_2, 85% N_2). *Arcobacter* species were grown in the same media but incubated under oxic conditions at 30ºC. Strains of the other genera were grown on nutrient agar at 37ºC for 24–48 h under oxic conditions.

Sample preparation and enrichment. Thirty commercial chicken products (10 carcasses, 10 livers, and 10 burgers) were purchased from five different retail markets in Valencia over a period of 3 months (one sample of each type and market per week, from April to June 2006). The samples were transported chilled to the laboratory and processed within 24 h of receipt. Samples (10 g) were individually homogenized for 2 min in a stomacher with 90 ml (1:10 dilution) of enrichment broth [Brucella broth containing cefoperazone, amphotericin B, and vancomycin antibiotic supplements (BioMérieux, Marcy l´Etoile, France)], and then incubated under microoxic conditions at 37ºC. To estimate the minimal period of time needed for selective enrichment, 1-ml aliquots of the broth cultures were taken at different time intervals (0, 24, and 48 h) for direct PCR, and a 10-µl loopful was streaked onto Campylosel agar plates (BioMérieux) for selective plating [5] at each time point. The plates were checked after 48–72 h of incubation at 37ºC under microoxic conditions for presumptive *H. pullorum* colonies, which appeared as pinpoint to 1 mm in diameter, translucent, watery, and weakly α-haemolytic after 3 days growth on blood agar. The colonies were plated onto Columbia blood agar and incubated under the same conditions. The resulting pure cultures were Gram-stained to assess their microscopic appearance and tested for indoxyl-acetate hydrolysis.

Conventional PCR assay for *H. pullorum*. Cells from an exponential-growth culture of each reference strain and presumptive isolates were harvested, washed twice with sterile PBS, and suspended in 500 µl TE buffer. One-ml aliquots from the samples grown in enrichment broth were centrifuged at 13,000 rpm for 10 min and the pellet was resuspended in 500 µl TE buffer. DNA was extracted and purified following the cetyltrimethyl-ammonium bromide (CTAB) method [26], and was stored at –20ºC until use.

H. pullorum was detected by PCR amplification using primers, designed by Stanley et al. [22], that target a 447-bp fragment of the *H. pullorum* 16S rDNA gene. Optimal PCR conditions were established with pure *H. pullorum* culture templates. The final reaction was carried out in a volume of 50 µl containing 5 µl of each sample, 0.5 µM of each primer (MWG-Biotech, UK), 0.2 mM of each deoxynucleotide, 2.5 mM $MgCl_2$, 1× PCR buffer, and 2.5 U of *Taq* polymerase (Ecogen, Barcelona, Spain). Samples were processed in a thermal cycler (Techne Progene, Cambridge, UK) as follows: initial denaturation at 95ºC for 5 min, 30 amplification cycles (denaturation at 94ºC for 30 s, annealing at 58ºC for 1 min, and extension at 72ºC for 1 min 15 s), and a final elongation step of 72ºC for 5 min. All the assays were performed in duplicate. Positive (DNA from *H. pullorum* NCTC 13153) and negative controls were included in all the assays. DNA from each of the reference strains was amplified to ensure the specificity of the reaction. PCR products (10 µl) were electrophoresed in a 1.2% (w/v) agarose gel in 1× Tris-acetate-EDTA buffer at 100 V for 60 min, stained with ethidium bromide, and viewed under UV light. A 100-bp ladder (MBI Fermentas, Burlington, Canada) was used to size the reaction products. To determine the theoretical sensitivity of the assay, an exponential growth culture (48 h) of *H. pullorum* NCTC 13153 was serially diluted into PBS buffer, yielding 10–10^8 colony-forming units (CFU)/ml. The number of cells at each dilution was calculated following plating on Columbia blood agar plates for 72 h; 1 ml of each dilution was analyzed as described above.

PCR identification of campylobacters. Isolates that were negative when tested with the conventional *H.-pullorum*-specific PCR assay were tested for *Campylobacter* spp. using the PCR assay described by Fermér and Engvall [9].

Real-time PCR assay for *H. pullorum*. DNA was extracted using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Indianapolis, IN, USA). Oligonucleotide primers to amplify a 180-bp 16S rDNA fragment for *H. pullorum* were: Hpull A (nucleotides 1201–1220), 5′-GAGCCAATCTCTAAAACATC-3′, and Hpull B (nucleotides 1261–1280), 5′-ACTAGTTTAGTATTCCGACT-3′. Primer specificity was verified using a BLAST search of GenBank (NCBI, Bethesda, MD, USA).

Optimal real-time PCR conditions were established. Each optimized reaction mixture consisted of 2 µl of DNA, 0.5 µM of each primer (TIB MOLBIOL, Berlin, Germany), 4mM $MgCl_2$, and 2 µl of LightCycler Fast-Star DNA Master SYBR Green I Mix (Roche Diagnostics) in a volume of 20 µl. The reactions were carried out in a LightCycler 2.0 (Roche) as follows: preliminary denaturation for 10 min at 95ºC, 40 cycles of denaturation at 95ºC for 3 s, annealing at 60ºC for 5 s, and primer extension at 72ºC for 4 s (slope 20ºC/s in all steps), with a single fluorescence acquisition step at the end of the extension. Melting-point analysis of the DNA product consisted of heating at 95ºC for 0 s and cooling to 70ºC for 20 s at a rate of 20ºC/s with continuous fluorescence acquisition. A final cooling step was done at 40ºC for 30 s (20ºC/s). Crossing threshold values (Ct) and efficiency of the amplification were determined with Roche LightCycler software 4.0. All the assays were conducted in duplicate. Positive (DNA from *H. pullorum* NCTC 13153) and negative controls were included in all assays. The specificity of

the real-time PCR assay was determined by testing all of the reference strains listed above. To determine the analytical sensitivity of the assay, a standard curve was generated by testing ten-fold serial dilutions (in PBS) of a 48-h culture of *H. pullorum*. Bacterial suspensions were quantified by spreading 100 µl of each dilution onto Columbia blood agar. The detection limit was expressed as CFU/ml of *H. pullorum*.

Sequencing of PCR products. To confirm the specificity of the PCR detection assays, three randomly selected PCR products obtained from carcass samples were purified with a Qiaquick PCR purification kit (Qiagen Iberia, Madrid, Spain) according to the manufacturer's instructions, and the products used for nucleotide sequencing. Both DNA strands were sequenced commercially (Sistemas Genómicos,Valencia, Spain). The sequences were analyzed using Chromas Version 1.43 (Technelysium, Tewantin Qld, Australia). The sequences obtained were compared to all *H. pullorum* and *H. canadensis* 16S rDNA sequences available in GenBank using BLAST software [http://www.ncbi.nlm.nih.gov/blast/].

Results

Evaluation of real-time PCR assay. The predicted 180-bp amplified DNA fragment from the *H. pullorum* reference strain had a melting temperature (Tm) of 86ºC, with Ct values ranging from 13 to 28 for different DNA concentrations. The efficiency of the assay, calculated from the second derivate maximum of each melt, was 1.777. No amplification was observed with DNA from any of the other reference strains except for *A. cryaerophilus* NCTC 11885, which had a melting temperature of 85.9ºC but only at Ct values > 35; it therefore was considered negative (Fig. 1).

The theoretical detection limit, defined as the lowest PBS dilution of *H. pullorum* cells consistently yielding a positive assay result, was 1 CFU/ml for the real-time PCR assay. However, for quantification purposes, the assay could not be used at that level because cell concentrations < 10^2 CFU/ml yielded inconsistent results. The detection limit of the conventional PCR assay for *H. pullorum* was 10 CFU/ml.

Culture of chicken sample isolates. After 72 h, cultures of the 30 food samples to be tested were all negative for *H. pullorum*, even with the inclusion of an enrichment step of up to 48 h. Isolates selected and cultured as putative *H. pullorum* but identified by PCR as *Campylobacter* species were not tested further.

PCR assays of chicken sample DNA extracts. Overall, 11 (37%) of the 30 DNA extracts prepared from chicken samples were determined to be positive for *H. pullorum*-like DNA by one or the other or both PCR assays. To confirm the results, each food sample was tested twice; for all samples, repeat PCR analysis yielded consistent results.

H-pullorum-like DNA was detected by real-time PCR assay in seven of the ten chicken carcasses, without need for enrichment; the characteristics of the amplicons reflected their melting-curves (Fig. 2A and 2B). These same samples also gave positive results by conventional PCR assay. All the other samples were negative prior to enrichment and remained negative when tested by PCR assays and by culture, even after 24 and 48 h of enrichment. To confirm the specificity of the real-time PCR, amplicons from assays of three chicken-carcass samples were purified and sequenced. All of them were 99% similar to the *H. pullorum* 16S rDNA gene sequence deposited in GenBank. Two of these sequences were also 96% similar to the same region in the *H. canadensis*

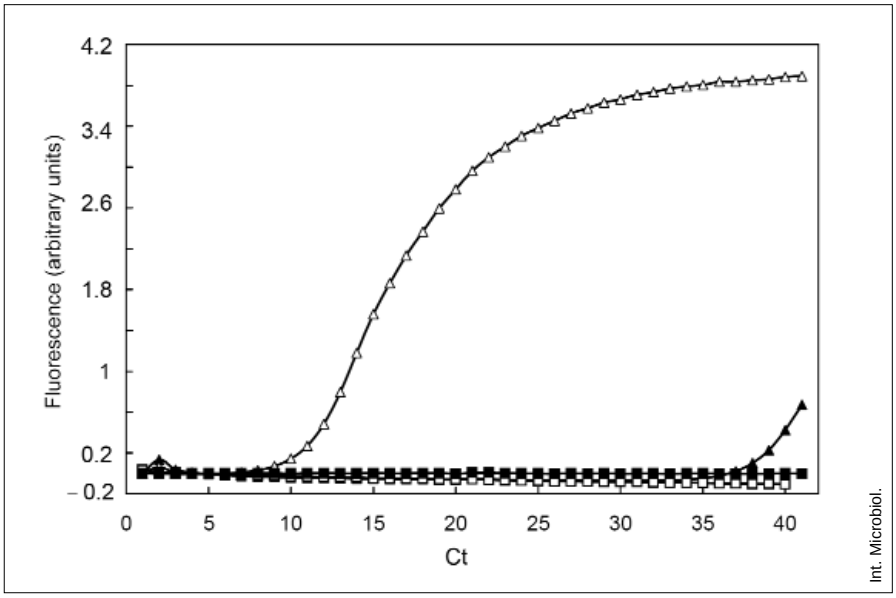

Fig. 1. Real-time PCR specificity assay: Amplification curves obtained from different reference strains. Hp, *Helicobacter pullorum* NCTC 13153; Ac, *Arcobacter cryaerophilus* NCTC 11885. Other lines indicate negative results from the reference strains other than *H. pullorum* that were included in the study .

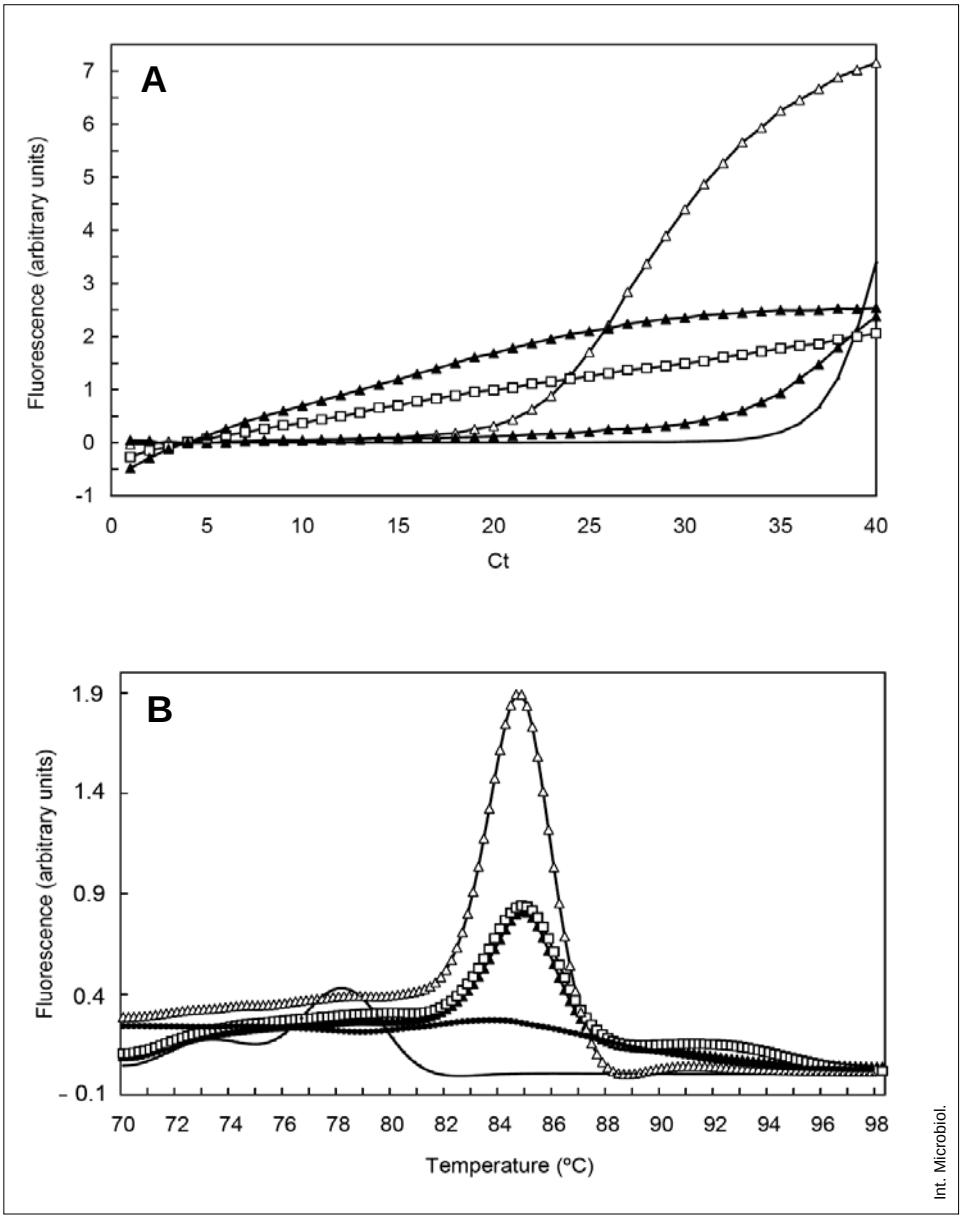

Fig. 2. (**A**) Real-time PCR results: Amplification curves obtained from different samples. Hp, *Helicobacter pullorum* NCTC 13153; 1, Carcass 10 at 24 h; 2, Carcass 6 at 24 h; C -, negative control. The other lines are of various negative samples. (**B**) Real-time PCR results: Melting peaks obtained from different samples. Hp, *H. pullorum* NCTC 13153; 1, Carcass 10 at 24 h; 2, Carcass 6 at 24 h; C-, negative control. The other lines are of various negative samples.

16S rDNA gene sequence deposited in GenBank. The other amplicon had <90% similarity to the *H. canadensis* sequence.

Two out of ten liver samples yielded positive results by conventional PCR after enrichment of the sample culture for 24 h, but results were negative by both methods after further incubation for 48 h. Another liver sample was weakly positive after 24 h only by real-time PCR, while after 48 h it was clearly positive by both conventional and real-time PCR.

Only one of the ten chicken-burger samples was positive for *H. pullorum*-like DNA without enrichment, as determined by conventional and real-time PCR assays; enrichment at 24 and 48 h resulted in loss of the amplification product.

Discussion

As microbiological food safety is of increasing concern, there is a growing need for sensitive, specific, and fast methods to detect zoonotic microorganisms in foods of animal origin [16,20]. *H. pullorum* has been linked to gastrointestinal disease but there is little information on its frequency in food. In this study, we investigated the presence of the bacterium in commercial chicken samples. All the samples tested were negative by culture, even with the inclusion of a 48-h enrichment step. This result was consistent with previous observations that *Helicobacter* species are difficult to isolate from foods [1,2,21].

As an alternative approach, PCR-based methods have become widely established for rapid detection and typing of microorganisms in foods [12,14]. To date, however, no such methods have been applied to the detection of *H. pullorum*. In our study, *H.-pullorum*-like DNA was detected in seven of the ten chicken carcasses tested. Although little information is available on the prevalence of *H. pullorum* in such samples, our results are consistent with previous reports [3]. The fact that *H. pullorum* colonies could not be isolated in any of these samples raises doubts about whether they contained viable cells. We believe this possibility to be unlikely as PCR detection was possible even after 48-h enrichment, and it has been reported that an enrichment step increases the level of viable cells whereas nonculturable or dead cells are diluted [8].

The detection rate of *H. pullorum* in burger samples proved to be low, with only one sample positive by both conventional and real-time PCR assays. Interestingly, detection was only possible without enrichment, perhaps due to the overgrowth of competitive microbiota. Alternatively, DNA but not viable bacteria may have been present in the sample.

Two chicken-liver samples (20%) were positive, but only by the conventional PCR assay and after 24 h of enrichment. As the bacteria could not be isolated by direct culture, the possibility of a false-positive result cannot be excluded. Another liver sample was weakly positive after 24 h by real-time PCR while after 48 h it was clearly positive by both PCR methods. This result suggests that a 48-h enrichment step might avoid some false-negative results. Although the small number of food samples included in our study limits its statistical signification, our detection rates were similar to those obtained by Ceelen et al. [6], who reported *H.-pullorum*-positive livers in two of the 11 flocks studied. The observed differences in the contamination rates between carcasses and livers could have been due to cross-contamination of broiler carcasses with cecal contents during poultry processing [3,10].

Our evaluation of the two PCR assays indicated that both were highly specific for *H. pullorum*. However, because a very similar organism, *H. canadensis*, has been described [25] the possibility that the positive results are attributable to that species needs to be considered. Although *H. canadensis* DNA was not included in our study, sequencing of three different sample PCR products revealed a similarity of 99% to the *H. pullorum* 16S rDNA gene sequence. Two amplicons were 96% similar to the same fragment of the *H. canadensis* 16S rDNA gene, but the other had < 90% similarity. For diagnostic purposes, it is generally accepted that partial 16S rDNA sequence similarities below 97% indicate a different species [13]. The fact that, to date, *H. canadensis* has not been reported in chicken products further supports the likelihood that the samples were contaminated with *H. pullorum*.

Both PCR assays can be considered highly sensitive. The minimal bacterial concentration consistently detected by real-time PCR was 1 CFU/ml, compared to 10 CFU/ml for the conventional PCR assay. However, at levels lower than 100 CFU/ml, the quantitative results of the real-time PCR proved irreproducible. Thus, the two methods were used only for qualitative analysis, with no marked difference in detection rates when they were applied to food samples.

In conclusion, our results, the first such reported study on food samples in Spain, suggest that *H. pullorum* is relatively common in chicken carcass samples in our geographical area. Further work is needed to validate the assay by obtaining positive cultures from food samples. Although conventional PCR is inexpensive to perform, real-time PCR is significantly faster and offers the possibility of screening more samples in an automated process. While the results confirm the inadequacy of culture methods in the detection of *H. pullorum*, both PCR protocols provide specific and sensitive techniques for assessing *H. pullorum* contamination of fresh chicken samples.

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