Development of PCR-based molecular tools for the detection of emerging food- ad water-borne pathogenic bacteria

L. Beneduce[*], **D. Fiocco** and **G. Spano**

Department of Food Sciences, Facoltà di Agraria, Università degli Studi di Foggia Via Napoli, 25, 71100 Foggia, Italy.

In the last decade, many foodborne and waterborne emerging pathogenic bacteria (eg. *Escherichia coli* O157:H7, *Helicobacter pylori*, *Campylobacter* spp.) were found to be a serious health hazard for both developed and developing countries. The use of molecular based technologies in microbial diagnostic has greatly enhanced the ability to detect and quantify pathogenic bacteria in food and water. Many of these molecular tools have been accepted and implemented in standard protocols for detection and quantification of the most important pathogenic bacteria, such as *Salmonella* spp. and *Listeria monocytogenes*. In this chapter we summarize the "state of the art" in this field of research and the experiences we had in our research activity, regarding the development of methods for detection of *E. coli* O157:H7 and *Helicobacter pylori*.

Keywords foodborne pathogens; waterborne pathogens; Real-time PCR

1. Introduction

Quality and risk assessment of food and water are the most important tasks for microbial diagnostic laboratories worldwide. The development of a global market for food industries and the intensive use of water resources make these tasks a major mission for researchers too. The discovery of new routes of transmission and the emerging of several foodborne and waterborne pathogenic bacteria revealed a serious health hazard for both developed and developing countries. The use of molecular based technologies in microbial diagnostic has greatly enhanced the ability to detect and quantify pathogenic bacteria in food and water. Many of these molecular tools have been accepted and implemented in standard protocols for detection and quantification of the most important pathogenic bacteria, such as *Salmonella* spp. and *Listeria monocytogenes*. Despite of rapid diffusion of molecular tools in microbiology laboratories, there are still many drawbacks and obstacles concerning specificity, reproducibility and reliability of nucleic acids and antibody based technologies for microbial detection. That leaded, to some extent, to an underestimation of those methods as a permanent alternative to conventional culture-based detection techniques that sill represent the "golden standard" for microbial diagnostic. The complexity of food and water matrices and the cross reaction of some molecular probes to target sites of innocuous bacteria closely related to pathogenic ones are the main enthralling challenges which researchers are still arguing with.

Researches in that field, conducted by our group at Department of Food Science, could be an interesting example of how to manage PCR methods for a reliable qualitative and quantitative detection of some emerging pathogenic bacteria in food and water.

2. Molecular tools for microbial diagnosis

Detection and identification of a microbial species in a complex sample like clinical, environmental and food matrices, is conventionally based upon isolation of the microrganism in pure culture and examination of its morphological and physiological properties. By this approach, microbiologists have been able to identify, characterize, type and quantify a wide range of microbial species. Notwithstanding

[*] Corresponding author: e-mail: l.beneduce@unifg.it, Phone: +39 (0)881 589 234

©FORMATEX 2007

the remarkable advancement in microbial diagnostics, particularly for pathogenic bacteria, the limits of conventional culture-dependent and phenotypic characterization are nowadays considered a severe constraint for precise, fast and reliable diagnostics. The ability of many bacteria to undergo a viable but not cultivable state, the significant shift in some biochemical properties of microrganisms when exposed to environmental stresses in the sample, the high virulence of some pathogenic bacteria (often below the threshold of sensitivity of conventional culture methods) are only brief examples of the restriction that traditional microbial techniques are not able to overcome.

With the scientific progress in the field of genetic and molecular biology, a variety of DNA, RNA and protein based methods have been developed, mainly in the field of clinical diagnosis. Microbiology "borrowed" most of those molecular tools in order to develop a new brand of detection and typing techniques for microrganisms. In the last 20 years there has been an extensive spread of DNA based methods that supplemented the conventional microbial techniques. Currently, most of these new tools are considered to be sufficiently developed and validated to replace classical microbial techniques.

The main advantage of microbial identification by genetic markers is the relative stability of the genotype rather than the phenotype. Nucleic acids and proteins can act as a "fingerprint" of a microbial species or strain, allowing a more accurate identification. DNA has all the characteristics of an ideal molecular marker:

a) is a relatively stable molecule; b) is present in all living organisms (except some viruses); c) contains genetic information; d) possess a semi-conservative structure of a double helix with complementary pairing of nucleotides; e) can be easily manipulated.

The development of methods such as hybridization and nucleic acid amplification allowed an impressive progress in the development of molecular markers. Above all, PCR (Polymerase Chain Reaction) is currently the most used technique because of its versatility, high specificity and sensitivity and its "open attitude". Using a PCR approach it is possible to selectively amplify a fragment of DNA that has a sequence shared only by particular species or strain. This selective reaction allows a magnification of the genetic information, up to a level that is easily detectable by the operator.

The "open attitude" of PCR is explained by the possibility of amplifying any desired portion of DNA selecting only the correct primer sequences able to bind with high specificity to target region of DNA chosen for the assay.

Recently, the development of quantitative real-time PCR has broaden the sensitivity and specificity and has allowed to quantify microbial cells in a given sample, coupling DNA amplification with continuous emission of fluorescence by DNA binding molecules (Sybr green) or DNA probes (Taqman, molecular beacon, etc.) during PCR reaction.

In the last 20 years many authors reported PCR and real-time PCR methods for identification and characterization of microrganisms in food and water. Most of those methods have been largely reviewed [1- 7]. On the other hand, few authors have focused the attention on the drawbacks and facilitations of nucleic acid amplification assays for microbial diagnosis [8].

3. Sampling and DNA extraction

3.1 sampling

To achieve the best results in a PCR assay it is crucial to take particular care during sampling. Conventional culture-dependent microbial techniques require aseptic condition during sampling and transportation and less time possible is needed before starting analyses. The advantage of PCR approach is that analyses can be performed also in a different day from sampling, using the precaution of freezing samples at -20 °C in order to avoid any loss of DNA quality and any growth of microrganisms in the sample during storage. This procedure is not appropriate when a pre-enrichment cultivation step is needed before PCR, in order to augment the number of target cells. In our experience the best way to facilitate sampling and to enhance DNA amplification is by storing the sample in sterile dark plastic bags and freezing it immediately while sampling, if possible. Otherwise, the use or refrigerated bags (4 °C) is

©FORMATEX 2007

opportune; in that case it's recommended to start with DNA extraction within few hours from sampling. When sampling water, it could be very useful to pre-filter the sample on nitrocellulose sterile filters (0,45 µm or 0,2 µm) and then freezing and storing the filter as a starting matrix for DNA extraction.

3.2 DNA extraction

Another critical step for successful PCR based techniques is the extraction and purification of an average quantity and good quality DNA. Nucleic acids extraction procedures are essentially based on three steps: 1) mechanical/chemical rupture of microbial cells, 2) precipitation/separation of non-nucleic acids substances (proteins, polysaccharides etc.) 3) precipitation and purification of nucleic acids.

Since molecular tools started to gain success in food and water microbiology, many DNA extraction procedures have been postulated. The critical evaluation of these procedures goes beyond the scope of this work, but several helpful research papers are recommended for deepening the argument [9-12].

Currently, many companies delivered functional, easy to use, DNA and RNA extraction kit that can overcome most of the troubles that may be encountered when operating with non-standard procedures.

To our experience the use of extraction kit (MoBio, Qiagen, Bio101 etc.) considerably increase the yield of DNA and reduces the co-extraction of inhibitors that can weaken the efficiency of nucleic acid amplification. Albeit, it's often necessary to make some custom adjustments to suggested extraction protocols, taking in consideration the peculiarity of food and water samples that have to be analyzed.

In our research experiences we were able to extract, purify and quantify bacterial DNA from raw and treated wastewater [13, 14]. The DNA was extracted from 20.0 ml of water sample, previously centrifuged at 300 g for 5 min to precipitate large particles. The supernatant was then removed and centrifuged at 19 000 g for 5 min to precipitate bacterial cells. Moreover, the pellet was double washed with 2.0 ml of sterile distilled water to reduce inhibitors for Taq polymerase. Furthermore, DNA was extracted following the protocol of a commercial kit. In that case the whole sample preparation and the DNA extraction procedure required <90 min.

Another approach we used for cell harvesting before DNA extraction procedures was based on filtration through sterile membranes. DNA from wastewater was extracted from 20 ml of sample. Samples were filtered through 0.45 µm pore size nylon membranes under vacuum filtration apparatus. Sterile TE (10mM Tris HCl pH 7.5 + 1mM EDTA) was used to wash membranes, during the filtration process. Membranes were folded and placed in a 15-ml sterile tube with 1-mm sterilized glass beads. After vigorous shaking for 5 min, the supernatant was transferred to a fresh tube, centrifuged at 13,000 rpm for 1 min, and the resulting pellet was double-washed with 2 ml of sterile TE to reduce inhibitors for Taq polymerase, and suspended in 1 ml sterile deionized water.

3.3 evaluating DNA yield and quality

An average quantity and quality of DNA extracted is essential to perform reproducible, sensitive and specific PRC reactions. To our experience, the best way to quantify DNA is by using more than one technique at the same time. For instance, it is possible to quantify DNA by measuring absorbance at 260 nm by a spectrophotometer. The quality of extraction can be evaluated by measuring the absorbance at 280 nm (to assess protein contamination) and 230 nm (to estimate the contribution of some buffers, like TAE and TE to total absorbance of sample). The ratio $A_{260/A280}$ and $A_{260/A230}$ should fall respectively between 1.8-2.0 and 1.9-2.5 when pure DNA is extracted. Additionally it could be useful to quantify DNA also by loading samples on agarose gel stained with ethidium bromide or Sybr green, together with a standard DNA and then comparing the fluorescence emitted by samples and standard by a software like Quantity one (Bio-rad). The latter method allows detection of double stranded DNA, the former is more qualitative (evaluation of eventual protein carryover in the sample) but cannot discriminate between single- and double-stranded DNA. A useful alternative can be the use of fluorogenic methods like Picogreen (Molecular Probes) capable of high sensitive quantification.

©FORMATEX 2007

4. Primers and probes for nucleic acid amplification

Sensitivity, specificity and reproducibility of a PCR-based assay to detect microrganisms in food and water are considerably dependent on the design of appropriate primers and probes. The use of 16SrDNA as a target gene for specifically detection of bacteria has been successful for most of the scopes in pathogenic species detection. 16SrDNA is universally considered the most suitable target gene because of its length (approx 1500 bp), is present in all bacteria in multiple copies and possesses 9 hypervariable regions (V1-V9) with considerable sequence diversity among different bacteria [15]. Unfortunately some pathogenic bacteria (like serotypes of enterohaemorragic *E. coli*) have a high similarity in all hypervariable regions, thus 16SrDNA was demonstrated to be not suitable for discriminating between different strains and serotypes. In our research works [13] we used primers and probes designed by other authors [16] on functional genes distinctive of enterohaemorragic *E. coli* O157:H7 , (*eae*, coding for intimin, and *stx1*, *stx2* coding for Shiga-like toxin). Those primers and probes (see below) allowed us a high sensitivity and reproducibility of PCR reaction, also if the specificity was limited because *eae* primers and probes could bind also to DNA from other serotypes like *E. coli* O157:NM and *E. coli* O55:H7 [17]. This experience demonstrates how difficult could be to find the appropriate target sequences for a suitable PCR method.

Designing primers and probes requires preliminary knowledge of the target gene, in terms of its sequence, number of copies present in the target microrganisms, inter-strain, intra- and inter-species variability of the gene sequence. Then the whole gene sequence has to be analyzed, in order to search for 15-25 bp segment of the sequence that present high variability between other related species of microrganisms. There is an ample variety of software able to find suitable sequences for primer design (e.g. Primer Express by Applied Biosystems, Oligo by BMI, and Primer Premier by Invitrogen).

When primer are evaluated "in silico" and designed, it is recommended to check their suitability in preliminary experiments with type strains of the selected target microrganism. In a recent work [14] we reported the successful design of a couple of primers able to detect *vacA* gene from *Helicobacter pylori*, useful to confirm the presence of particularly virulent strain of these pathogenic bacteria in water. Our primers were designed after retrieval of *vacA* sequences from NCBI database (http://www.ncbi.nlm.nih.gov/gquery/gque-ry.fcgi). Sequences were aligned and compared by ClustalW application (www.ebi.ac.uk/clustalw/), and the most conservative portions of these genes were analyzed with the soft-ware Primer Express 2.0 (Applied Biosystems, Forster City, CA-USA), in order to design a set of primers for both genes. Each primer sequence was evaluated "in-silico", aided by Blast software (http://www.ncbi.nlm.nih.gov/BLAST), to check specific pairing of our primers only with *H. pylori* gene sequences. Then we made some evaluation assays with 2 type strain of *H. pylori* (DSM 4867 and ATCC 700392) before applying the method with real-time PCR experiments.

5. PCR protocol assessment

When devising a PCR protocol it has to be taken into account that there are some key variables that can significantly affect the final results. A PCR reaction comprise the following components: target DNA, DNA polymerase, reaction buffer, $MgCl_2$, primers, dNTPs. Generally the concentration of DNA should fall between 1-100 ng/µl, primers 20-200 nM, dNTP 60-200 µM, DNA polymerase 0.5-1.5 U/µl. $MgCl_2$ concentration is adjustable, from 1.0 mM to 3.0 mM. Increasing $MgCl_2$ content in the reaction will enhance polymerase's activity but can also reduce the specificity by allowing the enzyme to bind and extend non-target DNA. The cycling programme that allows the PCR reaction to proceed is based on 25-40 temperature cycles as follows: initial DNA denaturation step (94 °C for 30 sec-1 min.), annealing step (40-60 °C for 30 sec-1 min.) and elongation step (72-74 °C for 30 sec-1 min.). The second step, during which primers bind specifically to DNA target sequence, is the critical one. Generally, annealing temperature should fall 4-5 °C below the melting temperature of primers. It is possible to increase annealing temperature, with the aim of limiting non-specific binding of primers to DNA. A preliminary evaluation of $MgCl_2$ content and annealing temperature is recommended for optimization of PCR method.

©FORMATEX 2007

6. post-PCR analyses

The evaluation of PCR output requires separation and visualization of amplified products by agarose gel electrophoresis. By electrophoresis it is possible to evaluate if the PCR fragment is of the expected size, based upon the distance between the two primers binding site in the gene sequence. Usually PCR fragments are chosen between 100 and 800 bp, so that the separation of fragments is optimal on a 0,8-2.0% agarose gel. A Molecular marker made of 10-15 dsDNA fragment of known size is added to the gel for evaluation of target fragment size. Ethidium bromide is the most used fluorescent dye that allows DNA to be visualized by UV light transilluminator (figure 1). Post-PCR analyses need some sample manipulation and are cumbersome and time-consuming. By real-time PCR the fluorescent dye (Sybr green) is added directly to the PCR reaction tubes and the fluorescence is measured during the amplification cycles.

Another advantage of Sybr green real-time PCR is the possibility of evaluating the specificity of the amplified product by melting curve analysis: at the end of PCR, a ramp of temperature is set, in order to denature the amplicons by temperature. When amplicons are denatured, the intercalating dye splits from DNA and a drop of fluorescence is measured. The temperature at which the target amplicons melt should correspond to the theoretical melting temperature, calculated on the basis of the target DNA sequence.

Another interesting real-time PCR application is the use of fluorescent probes: oligonucleotides are coupled with a fluorogenic dye that produces a signal during particular stage of amplificacion cycling programme. The signal is due to interaction of the probe with the target sequence. This approach has the advantage of a higher specificity, due to hybridization of the probe exclusively with target DNA.

In our experiments we used both Sybr green and Taqman probes and evaluated the reliability of both approaches to infer qualitative and quantitative information about target bacteria content in food and water samples. The detection of *Escherichia coli* O157:H7 in dairy and cattle wastewater has been evaluated with respect to conventional end-point PCR analyses (figure 2). As shown in figure 1, the limit of detection was 10^4 $CFUml^{-1}$ with PCR and increased to 10^3 $CFUml^{-1}$ by Taqman real-time PCR (figure 2.). We where then able to detect the pathogenic *E .coli* O157:H7 in wastewater previously resulted negative by culture methods and end-point PCR.

©FORMATEX 2007

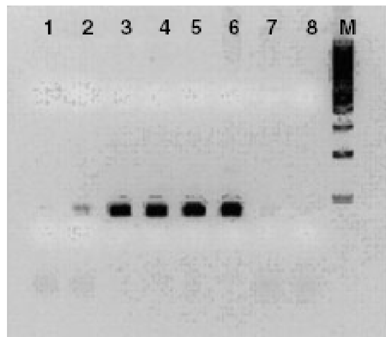

Fig. 1 PCR with *stx1* primers on DNA from wastewater samples spiked with increasing concentrations of *E. coli* O157:H7. Line 1, unspiked wastewater sample; line 2, wastewater spiked with 10^4 CFU ml^{-1}; line 3, wastewater spiked with 10^5 CFU ml^{-1}; line 4, wastewater spiked with 10^6 CFU ml^{-1}; line 5, wastewater spiked with 10^7 CFU ml^{-1}; line 6, wastewater spiked with 10^8 CFU ml^{-1}; line 7, DNA from *E. coli* strain Hb101 (negative control); and line 8, no template control.
All positive samples gave the expected 150 bp PCR product.

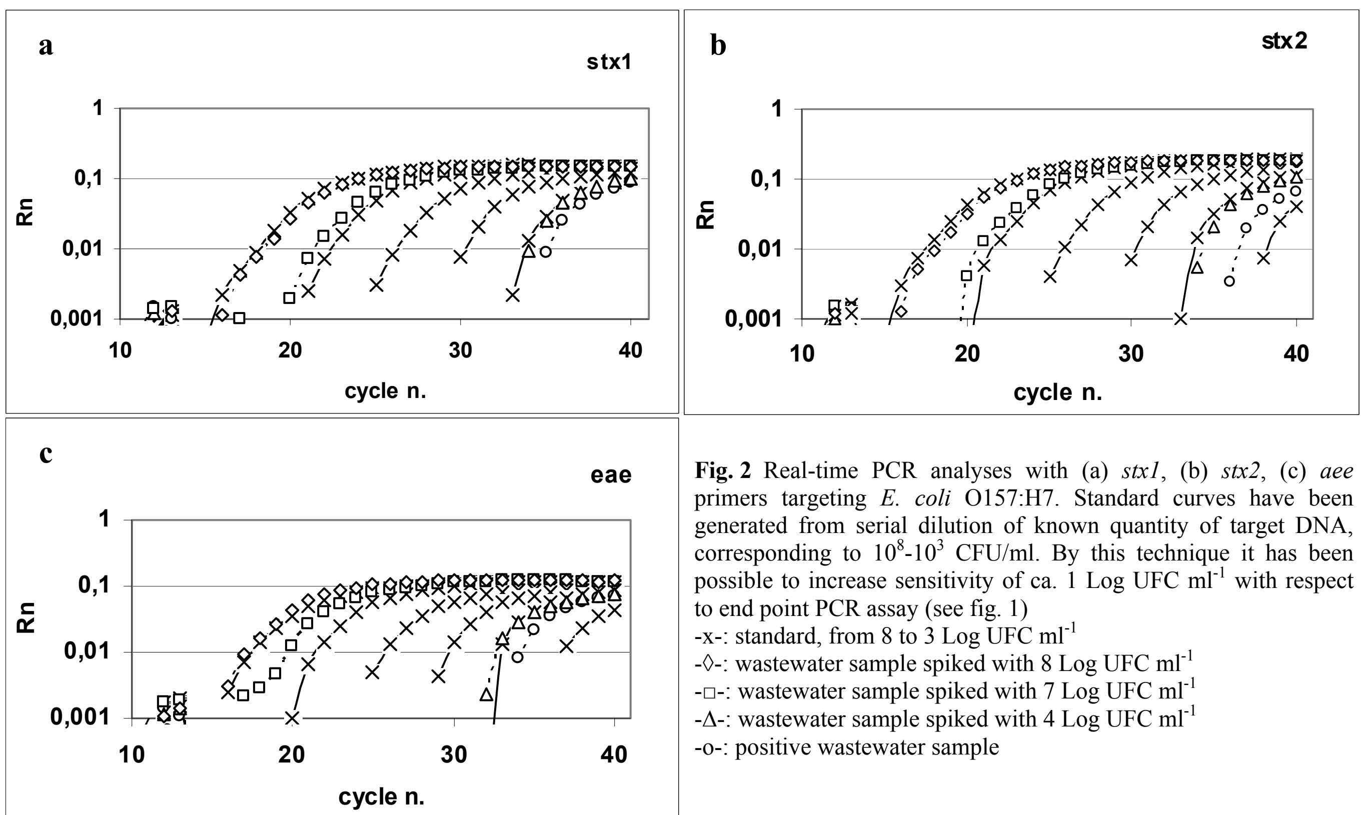

Fig. 2 Real-time PCR analyses with (a) *stx1*, (b) *stx2*, (c) *aee* primers targeting *E. coli* O157:H7. Standard curves have been generated from serial dilution of known quantity of target DNA, corresponding to 10^8-10^3 CFU/ml. By this technique it has been possible to increase sensitivity of ca. 1 Log UFC ml^{-1} with respect to end point PCR assay (see fig. 1)
-x-: standard, from 8 to 3 Log UFC ml^{-1}
-◊-: wastewater sample spiked with 8 Log UFC ml^{-1}
-□-: wastewater sample spiked with 7 Log UFC ml^{-1}
-Δ-: wastewater sample spiked with 4 Log UFC ml^{-1}
-o-: positive wastewater sample

When dealing with quantitative Real time-PCR, it's suggested to accurately evaluate the limit of detection and limit of quantitation. Above all, the evaluation of standard curve, generated by amplifying known quantities of DNA is mandatory, in order to validate the purposed method. During our experiments for quantification and detection of *Helicobacter pylori* in wastewater we generated fluorescence curves (threshold cycle *vs.* n. of cycle), and melting curves (-dFluorescence/dT *vs.* temperature). Both curves were analyzed aided by Microsoft Excel software (Microsoft Co., CA – USA). Standard curve (fig. 3) was developed by using serial dilutions of *H. pylori* strain DSM 4867 as internal standard, with DNA concentrations ranging from 10^4 to 10^{-2} pg μl^{-1}. Efficiency of amplification (E) was estimated from standard curve's data, with the formula: $E = (10^{-1/slope}) - 1$. A theoretical 100% efficiency reaction would have generated a slope of -3.32. The efficiency of amplification of total wastewater DNA

©FORMATEX 2007

extracted ranged between 90-96%, and the squared regression coefficient (correlations) for *ureC* gene was 0.99. When we used u*reC* primers, we were able to determine with a good approximation the quantity of *H. pylori* cells spiked in our samples, ranging from 10^7 to 10^3 cells ml^{-1}. Hence, the limit of quantification of the method is about 10^3 cells ml^{-1}, and that of detection 10^2 cells ml^{-1}. It's noticeable that in spite of a sensitivity of 1 Log CFU ml^{-1} stated by standard curve, our experimental data reports a limit of detection and a limit of quantitation respectively 1 and 2 Log higher, when dealing with DNA extracted from wastewater samples spiked with target bacteria. There are multiple reasons for this reduction in sensitivity: DNA is extracted from environmental matrix and not from pure culture, food and water samples are generally rich in other non-target microrganisms that may affect the amplification efficiency, very low level of target bacteria are still detectable but the information is not quantitative anymore, because of the high bias when dealing with trace amounts of target DNA.

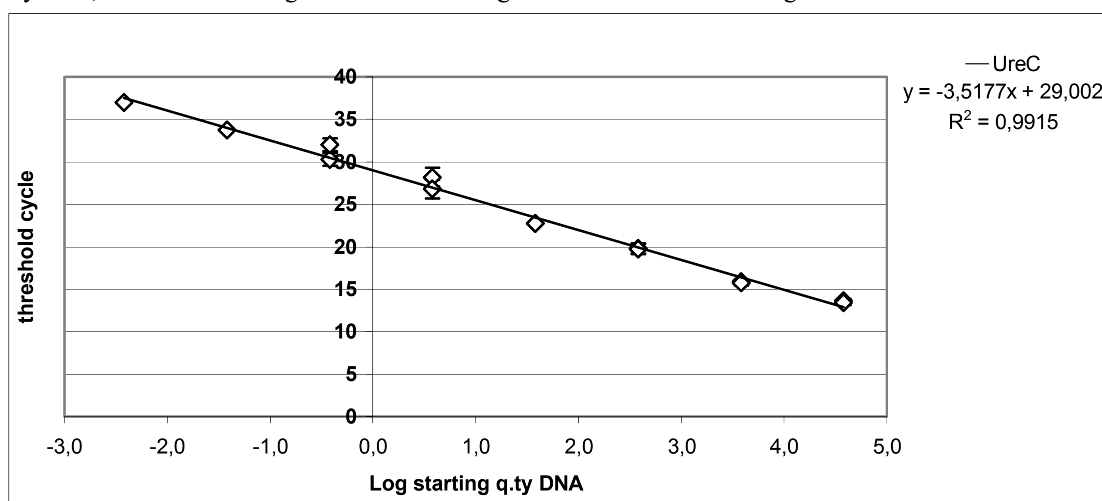

Fig. 3. Standard curve for the evaluation of amplification efficiency, limit of detection and limit of quantitation of *Helicobacter pylori* in water samples by Real.time PCR. The range of linearity is from 8 Log CFU ml^{-1} (corresponding to $5x10^4$ pg μl^{-1} DNA) to 1 Log CFU ml^{-1} ($5x10^{-2}$ pg μl^{-1} DNA).

7. Conclusions

The development of PCR based methods for detection and quantification of pathogenic bacteria in water and food has dramatically improved in the last few years. Moreover, the development of new laboratory tools for DNA extraction and manipulation and the enhancement of PCR reaction efficiency, allowed to increasing the specificity, sensitivity and reproducibility of PCR related assays.

In our research work we focused the attention on strategies for detection and quantification of *Escherichia coli* O157:H7 and *Helicobacter pylori*, two of the major water- and food-borne emerging pathogenic bacteria. Real-time PCR demonstrated his suitability as a powerful molecular tool for detection of low level of these pathogenic bacteria in complex environments like water and wastewater.

We were able to increase both the limit of quantitation and the limit of detection by 1 Log CFU ml^{-1} with respect to conventional culture methods and qualitative end-point PCR. However, the high virulence of these microrganisms requests further improvements in order to increase specificity and sensitivity of the technique.

©FORMATEX 2007

References

[1] R.A. McKee, C.M. Gooding, S.D. Garrett, H.A. Powell, B.M. Lund and M. Knox, Biochemical Society Transactions, 19, 698 (1991)
[2] J.E. Olsen, S. Aabo, W. Hill, S. Notermans, K. Wernars, P.E. Granum, T. Popovic, H.N. Rasmussen and O. Olsvik, International Journal of Food Microbiology, 28, 1 (1995)
[3] W.E. Hill, Critical Reviews in Food Science and Nutrition, 36, 123 (1996)
[4] P.M. Scheu, K. Berhof and U. Stahl, Food Microbiology, 15, 13 (1998)
[5] N.P. Rijpens and L.M. Herman, Journal of AOAC International, 85, 984 (2002)
[6] B. Malorny, P.T. Tassios., P. Radstrom, N. Cook, M. Wagner and J. Hoorfar, International Journal of Food Microbiology, 83, 39 (2003)
[7] J.L. McKillip and M. Drake, Journal of Food Protection, 67, 823 (2004)
[8] I. G. Wilson, Applied and Environmental Microbiology, 63, 3741 (1997)
[9] F. Petit, S. Craquelin, J. Guespin-Michel and C. Buffet-Janvresse, Research in Microbiology, 150, 143 (1999)
[10] J.E. Moore, J. Xuand and B.C. Millar, Journal of Microbiological Methods, 60, 131 (2005)
[11] C. Bonaiti, S. Parayre and F. Irlinger, International Journal of Food Microbiology, 107, 171 (2006)
[12] A.J. Fox, M.K. Taha and U. Vogel, FEMS Microbiolology Reviews 31, 84 (2007)
[13] G. Spano, L. Beneduce, V. Terzi, A.M. Stanca and S. Massa, Letters in Applied Microbiology 40, 164 (2005)
[14] L. Beneduce, G. Spano, A. Nabi, D. Fiocco, V. Terzi and S. Massa, Fresenius Environmental Bulletin, (FEB), 16, 749 (2007)
[15] S. Chakravorty, D. Helb, M. Burday, N. Connell and D. Alland, Journal of Microbiological Methods, 69, 330 (2007)
[16] A.M. Ibekwe, P.M. Watt, C.M. Grieve, V.K. Sharma and S.R. Lyons, Applied Environmental Microbiology, 68, 4853 (2002)
[17] V.K. Sharma and E.A. Dean-Nystrom, Veterinary Microbiology, 93, 247 (2002)

©FORMATEX 2007