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Evaluation of a Nested-PCR assay based on the phosphoglucosamine mutase gene (*glmM*) for the detection of *Helicobacter pylori* from raw milk

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ABSTRACT

Helicobacter pylori is an organism widespread in the human population and sometimes responsible for serious illnesses. Since *H. pylori* has been detected in Italy from an high percentage of sheep milk samples, it has been hypothesized that contaminated milk, may act as a vehicle of transmission of the microorganism to humans. In this work, a Nested Polymerase Chain Reaction approach has been used to detect *H. pylori* phosphoglucosamine mutase gene (*glmM*) from sheep, goat and cow milk artificially contaminated with wild *H. pylori* strains isolated from human gastric biopsies and the reference strain (*H. pylori* ATCC 43504). The technique showed a high sensitivity of 3 CFU/ml and proved to be both specific and rapid, The authors suggest that it could be used as a sensitive method for a rapid screening of sheep, goat and cow milk samples during the microbiological control of these large consumed foods.

1. Introduction

Helicobacter pylori is well known as the causative agent of gastritis and duodenal ulcer. Its presence on the human gastric mucosa has been accounted for several other diseases including gastric cancer, gastric lymphoma (mucosa-associated lymphoid tissue, MALT) and coronary heart disease (Edit, Stolte, & Tischer, 1994; Go, 2002; Mendall et al., 1994; Parsonnett et al., 1994; Wotherspoon et al., 1993). Indeed infections by *H. pylori* are considered a serious problem impairing the public health in both developed and developing countries (Brown, 2000).

However, the transmission pathways of *H. pylori* remain unclear. The most commonly acknowledged hypothesis is that infection takes place through fecal–oral route (Versalovic & Fox, 1999) and contaminated water and foods might play an important role in transmission of the microorganism to humans (Gomes & De Martinis, 2004; Meng & Doyle, 1997; van Duynhoven & de Jonge, 2001; Wesley, 1997).

In fact, *H. pylori* has been detected in drinking water (Glynn et al., 2002; Hegarty, Dowd, & Baker, 1999; Lu, Redlinger, Avitia, & Galindo, 2002; Queralt, Bartolomè, & Araujo, 2005), and in foods of animal origin, such as sheep (Dore, Sepulveda, Osato, Realdi, & Graham, 1999; Dore et al., 2001) and cow milk (Fujimura, Kawamura, Kato, Tateno, & Watanabe, 2002).

Furthermore *H. pylori* has been demonstrated to survive in complex foodstuffs like milk and ready-to-eat foods such as lettuce, tofu, and chicken (Fan, Chua, Li, & Zeng, 1998; Poms & Tatini, 2001; Quaglia et al., 2007). These data further support the hypothesis that food may act as a vehicle for *H. pylori* through primary contamination from animal reservoirs or secondary contamination due to unfit handling (human reservoir) (Quaglia et al., 2007).

Nowadays, only a few studies have been conducted in order to asses the presence of *H. pylori* in foodstuffs. This lack of data could be accounted for the difficulty in *H. pylori* isolation from foods, particularly in presence of a high load of accompanying microflora. In fact it is exacting and time-consuming since it requires the employment of selective media supplemented with numerous antibiotics, microaerophilic conditions and a long incubation periods (seven days) (Stevenson, Castillo, Lucia, & Acuff, 2000). Furthermore, *H. pylori* may produce viable nonculturable forms (VNC) (Cellini, Del Vecchio et al., 2004; Dunn, Cohen, & Blaser, 1997) not detectable by means of conventional microbiological techniques; however, it has been hypothesized that VNC forms are still infective (Bode, Mauch, & Malfertheiner, 1993; Cao, Li, Borch, Petersson, & Mardh, 1997) thus representing a potential microbiological risk for consumers.

Hence, several molecular studies have been performed in order to detect *H. pylori* in water and various foodstuff (Cellini, Del Vecchio et al., 2004; Quaglia et al., 2005; Velàzquez & Feirtag, 1999).

The *ureC* gene of *H. pylori* encodes for the phosphoglucosamine mutase catalyzing the interconversion of GlcN-6-phosphate (GlcN-6-P) and GlcN-1-P isomers (Mengin-Lecreulx & van Heijenoort, 1996) required for the biosynthesis of LPS and peptidoglycan. This gene has been shown to play an essential and unique role for *H. pylori* growth and survival (Bickley, Owen, Fraser, & Pounder, 1993; De Reuse, Labigne, & Mengin-Lecreulx, 1997; Labigne, Cussac, & Courcoux, 1991; Mengin-Lecreulx & van Heijenoort, 1996). It is

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presently designed as *glmM* rather than *ureC* since the production of the phosphoglucosamine mutase is unrelated to urease production (De Reuse et al., 1997).

A Nested Polymerase Chain Reaction (Nested-PCR) approach has been already employed for the detection of *H. pylori glmM* from seawater (Cellini, Del Vecchio et al., 2004). The aim of this study was to evaluate the sensitivity of the same Nested-PCR approach applied to artificially contaminated sheep, goat and cow milk.

2. Materials and methods

2.1. Bacterial strains

Two *H. pylori* strains (nat1 and nat2) from two human gastric biopsies samples and *H. pylori* ATCC 43504 (Promochem, LGC, UK), were used to artificially contaminate samples of tanked raw sheep, goat and cow milk collected in a local farm (Apulia region).

Isolation and identification of the strains nat1 and nat2 was performed slightly modifying the procedures described by Elizalde et al. (1998) (Dunn et al., 1997; Quaglia et al., 2007).

The nat1 and nat2 strains were subcultured on Wilkins–Chalgren anaerobe agar (Oxoid, Basingstoke, Hampshire, England) supplemented with 5% of defibrinated horse blood (Liofilchem, Teramo, Italy) and colistin methanesulfonate (30 mg/l), cycloheximide (100 mg/l), nalidixic acid (30 mg/l), trimethoprim (30 mg/l), and vancomycin (10 mg/l) (Sigma–Aldrich, Milano, Italy).

Lyophilized human strain *H. pylori* ATCC 43504 (Promochem) was reconstituted according to the supplier's instructions with 0.3 ml of brian heart infusion broth (BHIB) (Oxoid). This inoculum was added to 25 ml of BHIB supplemented with 5% of sterile horse serum (Sigma) in a 100 ml sterile flask and incubated for seven days at 37 °C with shaking under microaerophilic conditions (Anaerocult C mini, Merk, Darmstadt, Germany). The microorganism was cultured on Wilkins–Chalgren anaerobe agar (Oxoid) supplemented as described above.

After seven-day incubation at 37 °C under microaerophilic conditions (Anaerocult C mini, Merck), each strain (nat1, nat2 and *H. pylori* ATCC 43504) was harvested by a sterile cotton swab from the plates. The cells were washed three times with 2 ml of sterile phosphate-buffered saline (PBS, Sigma), pH 7.2, and then suspended in 10 ml of sterile saline solution (0.85% NaCl) separately.

One millilitre was used for the molecular identification of the strains nat1 and nat2 by conventional PCR, 1 ml was used for cell counting, while 3 ml were used for the artificial contamination of three groups (one per strain) of 9 ml of tanked raw sheep, goat and cow milk samples, obtained as described below (see Milk samples Section 2.4).

2.2. Molecular identification of nat1 and nat2 by conventional PCR

Nat1 and nat2 strains previously isolated and biochemically identified as *H. pylori* were molecularly processed as follows: a GenomicPrep Cells (Amersham Biosciences, GE Healthcare, Milan, Italy) was used to extract bacterial DNA from 1 ml of each suspension according to the supplier's instructions. For PCR test a master mix containing Buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), 200 μM of each dNTPs, 0.5 μM of each primer amplifying a 521 bp fragment of the 16S rRNA (Table 1) (Lu et al., 2002) and 1.25 U/50 μl of AmpliTaq Gold (Applied Biosystem, NJ, USA) was preliminary incubated at 94 °C for 10 min and then subjected to a 35-cycle amplification protocol at 95 °C for 1 min, at 58 °C for 1 min and at 72 °C for 1 min followed by an elongation step at 72 °C for 10 min (Quaglia, Dambrosio, Normanno, Parisi, & Celano, 2004). The PCR products were visualized under UV transillumination following electrophoresis on 1.5% agarose gel stained with ethidium bromide and using the Gene Ruler™ 100 bp DNA Ladder (MBI Fermentas, Milano, Italy) as a reference standard.

2.3. Bacterial count

In order to determine the *H. pylori* load of each suspension used for the contamination of the milk samples, serial dilutions up to 10^{-10} were made in sterile saline solution (0.85% NaCl) and 0.1 ml of each dilution was plated onto Wilkins–Chalgren anaerobe agar (Oxoid) supplemented as described above and incubated at 37 °C under microaerophilic conditions (Anaerocult C mini, Merck) for seven days.

2.4. Milk samples

Three 1000 ml samples of tanked raw sheep, goat and cow milk from a local farm (Apulia region) were used for the tests. The milk was put into sterile refrigerated containers (about 4 °C) and promptly delivered to our laboratory, where it was immediately tested.

A total aerobic mesophilic count in plate count agar (Oxoid) was performed for each milk sample on 10 ml incubated at 32 °C for 48 h. The milk was used for the experimental contaminations and to prepare the negative controls.

2.5. Negative controls

Several negative controls were prepared for each type of milk sample.

One consisted of uncontaminated sheep, goat and cow milk (each of 10 ml) that tested negative for *H. pylori* using a protocol recently described (Quaglia et al., 2007).

The other negative controls consisted of sheep, goat and cow milk samples artificially contaminated, following the procedure described below, with *Escherichia coli* (ATCC 25922), *Listeria monocytogenes* (ATCC 15313), and *Staphylococcus aureus* (ATCC 13565) cultured in Tryptone Soya Agar (Oxoid) for 24 h at 37 °C under aerobic condition, and *Campylobacter jejuni* subsp. *jejuni* (ATCC 29428), cultured in Columbia sheep blood agar (bioMèrieux, Rome, Italy) for 48 h under microaerophilic conditions (CampyGen, Oxoid). The bacterial cells were harvested from each plate and suspended in 2 ml of sterile saline solution (0.85% NaCl), separately. One millilitre of each suspension was used for the bacterial count, as detailed elsewhere (Harrigan, 1998), and 1 ml was used for the

Table 1
Primers used for the identification of the *H. pylori* strains (nat1, nat2 and *H. pylori* ATCC 43504) and for the Nested-PCR

Common name	DNA region (s) amplified	Primer sequence (5′–3′)	Amplicon size (bp)
NHP-F	16S rRNA	5′-GCAATCAGCGTCAGTAATGTTC-3′	521
NHP-R		5′-GCTAAGAGATCAGCCTATGTCC-3′	
Hp 1	*glmM* (*ure*C)	5′-AAGCTTTTAGGGGTGTTAGGGGTTT-3′	294
Hp 2		5′-AAGCTTACTTTCTAACACTAAACGC-3′	
Hp 3	21bp internal to primers	5′-CTTTCTTCTCAAGCGGTTGTC-3′	252
Hp 4	Hp 1 and Hp 2	5′-CAAGCCATCGCCGGTTTTAGC-3′	

contamination of 9 ml of sheep, goat and cow milk samples, separately.

2.6. Nested-PCR

Bacterial DNA from 1 ml of each artificially contaminated sheep, goat and cow milk sample was extracted with a DNeasy Tissue Kit (QIAGEN, Milano, Italy) slightly modifying the supplier's instructions. Briefly, after centrifugation at 15,400*g* for 10 min, 180 μl of alanine aminotransferase buffer and 20 μl of lysozime (Sigma) were added to the pellet and incubated at 37 °C for 20 min.

Oligonucleotide primers Hp 1 and Hp 2 were used to amplify a 294 bp PCR product of the *H. pylori glmM* (*ure*C) gene (Bamford, Lutton, O'Loughlin, Coulter, & Collins, 1998) (Table 1). Internal primers Hp 3 and Hp 4 were used to amplify a 252 bp region located 21 base pairs downstream and upstream to primers Hp 1 and Hp 2, respectively (Bamford et al., 1998) (Table 1). The primers were synthesized by Tib Molbiol (Tib Molbiol S.r.l., Genoa, Italy).

Samples (2 μl) of each extract were amplified in 50 μl of reaction mixture containing 10 × HotMaster Taq buffer (10 mM Tris-HCl, pH 8.5, 50 mM KCl, 25 mM $MgCl_2$), 200 μM each of the dNTPs, 0.5 μM of each primer (Hp 1 and Hp 2), and 1.25 U of Hot Master Taq DNA polymerase (Eppendorf AG, Hamburg, Germany). PCR amplification was performed according to the following protocol: 95 °C for 2 min and 33 cycles at 94 °C for 1 min, 61 °C for 2 min, 72 °C for 1.5 min followed by 72 °C for 5 min. After the amplification, 1 μl of the final product was transferred in a second step reaction mixture and re-amplified for 30 cycles using primers Hp 3 and Hp 4 under the following conditions: 95 °C for 2 min and 30 cycles at 94 °C for 1 min, 62 °C for 2 min, 72 °C for 1.5 min followed by 72 °C for 5 min. Positive control reaction consisted of *H. pylori* genomic DNA from type strain ATCC 43504 (Promochem).

The Nested-PCR products were visualized under UV transillumination following electrophoresis on 1.5% agarose gel stained with ethidium bromide and using the Gene Ruler 100-bp DNA Ladder (MBI Fermentas, Milano, Italy) as a reference standard.

Each test was repeated three times.

2.7. Sensitivity of the Nested-PCR

To evaluate the sensitivity of the technique, the Nested-PCR was performed on 10-fold dilutions (up to 10^{-10}) of the raw sheep, goat and cow milk samples artificially contaminated with the *H. pylori* ATCC 43504 strain. The dilutions were performed in raw sheep, goat and cow milk, and bacterial DNA was extracted from each dilution.

2.8. Specificity of the Nested-PCR

To evaluate the specificity of the technique, the Nested-PCR was performed on the uncontaminated sheep, goat and cow milk samples and on each milk samples contaminated with the bacterial strains used to prepare the negative controls.

3. Results

The PCR assays performed on the strains nat1 and nat2 yielded amplicons of 521 bp in length as expected (Lu et al., 2002).

The mean value of bacterial counts for the *H. pylori* suspension was determined to be 1.7×10^8 CFU/ml for nat1 strain, 2.3×10^6 CFU/ml for nat2 strain and 3×10^7 CFU/ml for *H. pylori* ATCC 43504.

The total aerobic mesophilic count for the tanked sheep, goat and cow milk samples was 1.3×10^5 CFU/ml, 1.7×10^7 CFU/ml and 2×10^4 CFU/ml, respectively.

All of the Nested-PCR assays that were performed on the artificially contaminated raw sheep, goat and cow milk amplified a final product of 252 bp.

The Nested-PCR assays performed on all the negative controls were consistently negative.

The final product of 252 bp was visualized up to the 10^{-7} dilution (3 CFU/ml) for all tests performed in order to assess the sensitivity of the method on artificially contaminated raw sheep, goat and cow milk.

4. Conclusion

H. pylori has been estimated to infect about 50% of the world's population at early age (Frenck & Clement, 2003; Guillermo, Rothenbacher, & Brenner, 2004; Torres et al., 2000). Despite of this record the precise mode of transmission to humans is still unknown. Foodstuff has been considered as the most likely source of infection (Wesley, 1997; Meng & Doyle, 1997; Velàzquez & Feirtag, 1999) since raw sheep and cow milk have been found contaminated with *H. pylori* (Cohen, 1996; Dore et al., 2001; Dunn et al., 1997; Fujimura et al., 2002; Hultèn et al., 1996; van Duynhoven & de Jonge, 2001; Wesley, 1997).

However, since isolation of *H. pylori* from foods is extremely difficult due to the presence of accompanying microflora and to the presumably very low *H. pylori* load contaminating foodstuff (Cellini, Del Vecchio et al., 2004; Dunn et al., 1997; Stevenson et al., 2000), it is important to evaluate specific, sensitive and rapid methods for the detection of this pathogen from food products, and in particular from milk.

This article reports the results of the employment of a Nested-PCR assay for the detection of *H. pylori* in raw sheep, goat and cow milk samples. In a previous work, Cellini, Del Vecchio et al. (2004) applied the same procedure to assess the presence of free and plankton-associated *H. pylori* in seawater.

The set of primers employed specifically amplify a region of the phosphoglucosamine mutase *glmM* gene that is always present in all *H. pylori* strains (Kansau et al., 1996), and it is previously reported to improve sensitivity of the *H. pylori* detection from samples containing both prokaryotic and eukaryotic cells as well as many organic impurities (Bamford et al., 1998).

The method has proven to be rapid (about 7 h) specific and sensitive. The specificity of the method has been assessed on samples of raw sheep, goat and cow milk artificially contaminated with foodborne pathogens commonly found in these types of milk (Barbuddhe, Malik, Bhilegaonkar, Kumar, & Gupta, 2000; Normanno et al., 2005, 2007; Vautor, Abadie, Guibert, Huard, & Pèpin, 2003). The primer set here employed did not amplify other genetic material from the uncontaminated control samples nor from the samples artificially contaminated with others bacteria. The specificity of the technique here described has to be considered particularly high since raw milk samples artificially contaminated with *C. jejuni* (phylogenetically tightly related to *H. pylori*, see Wesley, 1997) tested also negative.

The sensitivity of the Nested-PCR technique registered in this survey (i.e. 3 CFU/ml detectable from all types of milk artificially contaminated) is higher than that previously recorded by Cellini, Del Vecchio et al. (2004) in seawater samples (i.e. limit of detection of 62 CFU/ml).

In addition this assay showed to be more sensitive and specific than other methods described for *H. pylori* detection from water and food samples, such as microbiological (Poms & Tatini, 2001), immuno-separation (IMS) followed by PCR, autoradiography (Cohen, 1996) and ATP bioluminescence (Nilsson, Aleljung, Nilsson, Tyszkiewicz, & Wadström, 1996; Shahamat, Mai, Paszko-Kolva, Kessel, & Colwell, 1993). In fact, while the microbiological method

has been proven to be highly selective for the recovery of *H. pylori*, it lacks of sensitivity in recovering very low numbers of *H. pylori* cells (Poms & Tatini, 2001); the IMS assay followed by PCR on the 16S rRNA, although is a good method because it allows to concentrate the microorganism from foods, it appeared expensive, exacting and time-consuming (Cohen, 1996). The last two methods, autoradiography, ATP bioluminescence although successfully employed for the detection of *H. pylori* from water, human stool and pure culture, have never been tested on food samples (Nilsson et al., 1996; Shahamat et al., 1993; Velàzquez & Feirtag, 1999). In addition, the ATP bioluminescence assay does not allow to distinguish among ATP from different cell sources when applied to a complex system such as foodstuff (Schram, 1991).

Recently, a Multiplex touchdown PCR (MT-PCR) for the detection and characterization of *H. pylori* from sheep milk has been described; although this assay proved to be highly specific, the Nested-PCR technique described here showed to be more sensitive (3 CFU/ml vs. 15 CFU/ml) (Quaglia et al., 2005).

Finally, the Nested-PCR method for the detection of *H. pylori* from raw sheep, goat and cow milk might represents a rapid and sensitive screening tool to be employed in routinely milk sanitary controls.

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