Prevalence and detection of *Helicobacter pylori* in raw cows' and goats' milk in selected farms in the Czech Republic

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Summary

Helicobacter pylori is currently found in more than a half of the human population and is the cause of serious gastrointestinal diseases such as gastritis, gastric ulcers or adenocarcinoma. *H. pylori* infection can be transmitted to humans through contaminated raw materials and food of animal origin. In this study, individual cows' milk samples (n = 77) and individual goats' milk samples (n = 52) from two farms in Moravia (Czech Republic) were analysed. The classical culture method and the nested polymerase chain reaction (nested PCR) method were used for detection of *H. pylori.* The culture method did not demonstrate the presence of *H. pylori* in any of the examined samples. Using the nested PCR method, 31 samples (40 %) of raw cows' milk and 30 samples (58 %) of raw goats' milk were positive for the presence of *H. pylori*. The study demonstrated that nested-PCR was highly sensitive for the detection of *H. pylori* in raw milk samples. The results of the study showed the presence of *H. pylori* DNA in a high percentage of both cows' and goats' raw milk samples, indicating a high possibility of inter-animal transmission within individual farms.

Keywords

Helicobacter pylori; nested polymerase chain reaction; food safety; raw milk

Helicobacter pylori is a gram-negative, microaerophilic, motile bacterium colonizing the gastric mucosa. Infection with *H. pylori* is directly related to the occurrence of serious human diseases such as chronic gastritis, duodenal ulcer or gastric cancer. In 1994, International Agency for Research on Cancer (IARC), a subordinate organization of World Health Organization (WHO), identified *H. pylori* as a „group 1 (definite carcinogen)" [1]. The routes of transmission and reservoirs of this bacterium are topics that have been the subject of much research but have not yet been precisely elucidated. The findings of *H. pylori* in food of animal origin, vegetal food and water support the hypothesis of many authors that the bacterium is a food-borne pathogen [2].

Milk from various species of animals is an important food consumed worldwide by people of all ages. In many parts of the world, it is a staple food, forming an integral part of the daily diet. If milk is one of the routes of transmission of *H. pylori*, this finding may be considered in the future, for example, in the search for the origin of infection in patients. The results and conclusions obtained in this study can be used, for example, to determine the origin of helicobacteriosis in a human population.

Since the discovery of *H. pylori*, the classical culture method has been used as a routine diagnostic test in human medicine, being considered the "gold standard" among individual methods. *H. pylori* is a fastidious bacterium requiring nutrient-rich media, microaerophilic environment, high humidity (96–100 %) and pH close to 7.0. The media are usually enriched with blood (as a reducing agent) and with specific antibiotics. The

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culture method has been used in several studies to determine the presence of *H. pylori* in milk. Published studies indicated that the method was not optimal for examination of foods [2–4]. On the other hand, high sensitivity was demonstrated in the case of molecular-biological methods allowing specific sequential amplification of DNA of the microorganism together with molecular typing of the strains. Various polymerase chain reaction (PCR) methods were developed for identification of *H. pylori* in food, including multiplex PCR, nested PCR, real-time PCR and reverse-transcription PCR [2].

The aim of this study was to find the most suitable method for detection of *H. pylori* in milk, especially by using and comparing methods based on both the classical culture procedure and molecular-biological detection, specifically, the nested PCR method. Another aim of the work was to obtain data regarding the occurrence of *H. pylori* in raw cows' and goats' milk samples from farms in the Moravian region of the Czech Republic. This may help to evaluate the significance of raw milk as a possible route of transmission and thus a source of *H. pylori*.

MATERIAL AND METHODS

Samples

Individual cows' milk samples ($n = 77$) and individual goats' milk samples ($n = 52$) from two farms in Moravia (Czech Republic) were analysed. The cows' milk samples were obtained during machine milking by dripping the milk into a special sampling cylinder. After milking, the cylinder was turned twice to mix the sample and the sample was discharged into a sterile sampling tube through a valve. Individual goats' milk samples were obtained by hand milking into sterile sampling tubes. The samples were transported and stored at 4–6 °C. The samples were examined in the laboratory within 24 h after collection.

Culture method

Direct culture of the samples was performed using *Helicobacter* agar prepared from the following ingredients: brain heart infusion broth (Oxoid, Basingstoke, United Kingdom), Norit A (Electrophoresis, Heidelberg, Germany), potato starch (Sigma-Aldrich, St. Louis, Missouri, USA), yeast extract (Oxoid), agar bacteriological (Oxoid), defibrinated horse blood, horse serum, Enrichment A (Dulab, Dubné, Czech Republic) and 0.5 ml mixture of antibiotics, containing vancomycin (30 000 IU·ml^{-1}; Mylan Pharmaceuticals, Prague, Czech Republic), colistin (30 000 IU·ml^{-1}; Teva Pharmaceuticals, Prague, Czech Republic) and trimethoprim (1 500 IU·ml^{-1}, Sigma-Aldrich). The plates were incubated at 37 °C for 9 days in an anaerostat under microaerophilic conditions (5 % O_2, 15 % CO_2 and 80 % N_2) created using a CampyGen 2.5L developer (Oxoid). After incubation, suspect colonies (small, clear, transparent and dome-shaped) were subjected to the urease assay UREASAtest50 (TestLine Clinical Diagnosis, Brno, Czech Republic) to detect *H. pylori* urease activity.

Nested PCR

In this reaction, two pairs of primers are used, i. e. a pair of external primers and a pair of internal primers. The internal primers attach to the sequence that is bordered by the external primers. The reaction with the external primer pair is performed first and the resulting product is used as a template for the reaction with the internal primers. This arrangement increases sensitivity. Further, combining two primer pairs also increases specificity. *H. pylori* DNA was directly extracted from milk using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the instructions provided by the manufacturer. Oligonucleotide primers for PCR were those published by Quaglia et al. [5]. The external oligonucleotide primers were Hp1 (5'-AAG CTT TTA GGG GTT AGG GTT T-3') and Hp2 (5'-AAG CTT ACT TTC TAA CAC TAA ACG C-3') targeting *glmM* gene of *H. pylori* with the size of the amplification product of 294 bp. The internal primers were Hp3 (5'-CTT TCT TCT CAA GCG GTT GTC-3') and Hp4 (5'-CAA GCC ATC GCC GGT TTT AGC-3'), which amplified the internal part of *glmM* gene with the size of the amplification product of 254 bp. Primers were obtained from Generi Biotech (Hradec Králové, Czech Republic). The reaction mixture was prepared using PPP Master mixture (Top-Bio, Vestec, Czech Republic), water for PCR (Top-Bio), Hp1 (0.5 μmol·l^{-1}) and Hp2 (0.5 μmol·l^{-1}). Then, 2 μl of extracted DNA solution was added to 23 μl of this mixture. The nested PCR was performed using a thermocycler MJ Research PTC-200 Thermal Cycler Dual 48 (Marshall Scientific, Hampton, New Hampshire, USA) using a temperature programme involving initial denaturation at 95 °C for 2 min, followed by 33 cycles (94 °C for 1 min, 61 °C for 2 min, 72 °C for 1.5 min) and the final synthesis at 72 °C for 5 min. After completion of the first amplification, 2 μl of its product was added to a second reaction mixture of the same composition as the first reaction, with the only difference that

the added primers were Hp3 and Hp4. The temperature programme involved initial denaturation at 95 °C for 2 min, followed by 30 cycles (94 °C for 1 min, 62 °C for 2 min, 72 °C for 1.5 min) and the final synthesis at 72 °C for 5 min. Electrophoretic separation at 120 V, 90 mA for 60 min in 1.5% agarose gel (Serva, Heidelberg, Germany) stained with ethidium bromide followed by visualization under UV-light was used to detect PCR products. DNA ladder of 100–4 000 bp (Lonza, Rockland, Maine, USA) was used as a molecular size standard. Positivity was indicated by the presence of a DNA fragment of 254 bp. *H. pylori* DNA from a strain obtained from a human gastric mucosal sample was used as a positive control. This *H. pylori* control strain was provided by the Microbiology Institute of St. Anne's University Hospital (Brno, Czech Republic).

RESULTS AND DISCUSSION

The use of the culture method for detection of *H. pylori* in food is challenging and tedious, as it requires selective media with antibiotics, microaerophilic conditions and long culture times of up to 9 days. Another disadvantage of the culture method consists in the inability to capture the coccoid, viable non-culturable form (VNC) of the bacterium [6]. In our study, the presence of *H. pylori* was not detected using the culture method in any of the samples examined (data not shown). In addition to the reasons previously mentioned, unfavourable conditions between sample collection and examination (transport of samples to the laboratory and storage at refrigerated temperatures and under aerobic conditions) may have impacted the growth of the bacterium. Milk is also a food rich in accompanying microflora, which can significantly suppress the growth of *H. pylori* on agar media. The competing microorganisms in milk samples is often regarded as one of the main factors that prevent the detection of *H. pylori* by the classical culture method [2, 7]. The culture method has been used in practice for the detection of *H. pylori* in biopsy samples, but it has not proven to be very suitable for food samples. The inability to culture *H. pylori* from raw milk was found previously [8, 9]. In their study, Fujimura et al. [10] were only able to culture *H. pylori* in 1 out of 13 milk samples, although all were confirmed as positive by semi-nested PCR. Currently, there are no standardized culture methods available to guarantee the isolation of *H. pylori* from samples rich in associated microflora, such as food samples or, in our case, milk samples [2]. Moreover, the isolation procedure, although allowing the isolation of *H. pylori* from various foodstuffs and found to be highly selective for *H. pylori*, was already demonstrated to lack sufficient sensitivity to reproducibly recover very low numbers of *H. pylori* (10 CFU·ml^{-1} of liquid samples to recover at least one colony per plate) [11]. Nevertheless, the sensitivity of the nested PCR approach (3 CFU·ml^{-1}) [12] allowed to overcome the constraints of the above microbiological method. The sensitivity of the nested PCR technique was determined by Quaglia et al. [5]. In their study, nested PCR for detection of the *H. pylori* gene *glmM* was performed in a series of decimal dilutions (up to 10^{-10}-fold) of the raw ewes', goats' and cows' milk artificially contaminated with the reference strain *H. pylori* ATCC 43504. The technique showed a high sensitivity of 3 CFU·ml^{-1}. Although PC- based methods do not discriminate between living and dead microorganisms, they appear to be very helpful as a method of screening of samples [5].

In our study, of the 77 raw cows' milk samples, 31 (40 %) were determined to be positive for *H. pylori* by the nested PCR method (data not shown). Out of the 52 individual raw goats' milk samples analysed by this method, 30 (58 %) were found to contain *H. pylori* DNA (data not shown). An illustration of a result of nested PCR to detect *H. pylori* in raw goats' milk is given in Fig. 1.

When comparing the results of this work with those of other studies, there is quite a significant difference in the proportion of positive goats' milk samples. Talaei et al. [13] investigated the occurrence of *H. pylori* in cows' and goats' milk in a region of Iran with the use of the 16S rRNA PCR analysis for the detection of *H. pylori* directly in DNA isolated from samples. Detection limit of this method was as little as 0.1 pg·μl^{-1} of DNA, which is approximately 10-fold the detection limit of the nested PCR method [14]. This probably explains the low positivity rate of 12/75 (16 %) for cows' milk and 2/42 (4.8 %) for goats' milk samples. The sensitivity of PCR and nested PCR for *H. pylori* detection was studied by Ho et al. [14] using decimal serial dilution of *H. pylori* DNA (from 1 ng·μl^{-1} to 1 fg·μl^{-1}) using primers targeting a selected region of 16S rRNA gene. Sensitivity was found to be affected by the number of cycles used in the reaction. DNA amplification by conventional PCR with 30 cycles permitted the detection of 10 pg of starting bacterial DNA, while increasing the number of cycles to 40 improved the sensitivity of detection to 0.1 pg·μl^{-1} of DNA. With the use of nested PCR, 0.01 pg·μl^{-1} of DNA was detectable. In nested PCR used in this

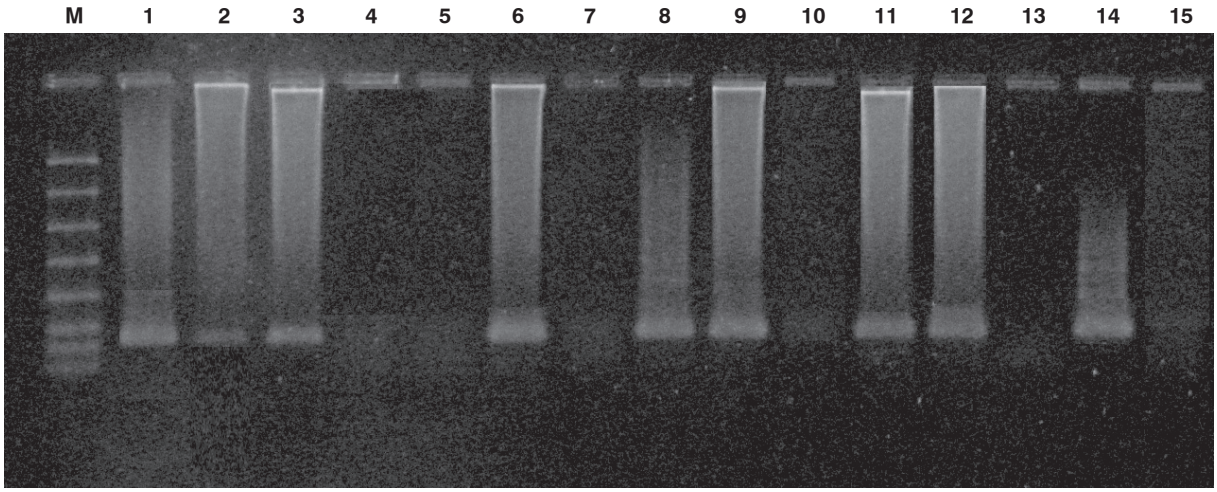

Fig. 1. Gel electrophoretic separation of the products of nested PCR for detection of *Helicobacter pylori*.

Lines: M – molecular size standard, (1–13) – samples of raw goats' milk, (14) – positive control, (15) – negative control. Lines 1, 2, 3, 6, 8, 9, 11, 12, 14 – positive samples.

study, a total of 50 cycles was used (first reaction – Hpl-Hp2: 25 cycles, second reaction – Hp3-Hp4: 25 cycles) [14].

Mousavi et al. [15] detected the presence of *H. pylori* in 20/120 (16.6 %) samples of cows' milk and in 28/100 (28 %) samples of goats' milk using a culture method and subsequent PCR confirmation. This is a very surprising result, as in most of the studies already mentioned, the culture method was unsuccessful at detection of *H. pylori* in food samples. Rahimi et al. [16] used conventional PCR to detect the *glmM* gene and found *H. pylori* present in 14.1 % and 8.7 % of cows' and goats' milk samples, respectively. Using the nested PCR method, Quaglia et al. [12] detected the presence of *H. pylori* in 25.6 % of raw goats' milk samples. By this method, which has also been used in our work, it is possible to detect 100 CFU·ml^{-1} [12]. The high percentage of raw goats' milk samples positive for *H. pylori* in this study (58 %) may be due to the origin of the samples. All of them came from the same farm where the animals were in daily contact with each other, and it was therefore possible that *H. pylori* was transmitted from animal to animal or by contaminated bedding. According to the available information, the sources of *H. pylori* may include not only animal bedding but also the ambient dust and contaminated water. Animals also have a feeding trough during milking, through which the bacteria may be spread via the saliva of infected animals. Another possible explanation for such a high detection rate could lie in the contamination of milking equipment and subsequent transmission to other animals [9, 10].

Cows' milk showed a lower percentage of samples positive for *H. pylori* compared to goats' milk, yet this percentage was still relatively high compared to some other studies [13–15]. For example, Bianchini et al. [7] investigated the occurrence of *H. pylori* in tank samples of cows' milk from herds originating from the northern Italian region. Out of 163 samples, they determined only 3 samples as positive for the presence of the Helicobacteriaceae using the nested PCR method targeted to *16S rRNA* gene. Samples positive for Helicobacteriaceae were confirmed by PCR targeting the *23S rRNA* gene of *Helicobacter* and further tested for the presence of *H. pylori* using the nested-PCR method described by Quaglia et al. [12]. Of those three positive Helicobacteriaceae samples, none of them was confirmed as *H. pylori*. This low positivity level can be explained by the high dilution of the milk in the storage tank, where the possibility of detection is reduced compared to individual milk samples. On the other hand, in a study by Quaglia et al. [12], the presence of *H. pylori* was detected in raw cows' milk in a region of southern Italy and their results showed 50 % positive samples. This was also confirmed by a study using semi-nested PCR for detection of *ureA* gene [10], in which the percentage of positive samples of raw cows' milk collected in various parts of Japan was as high as 72 %. The different results of these studies can be attributed to the area from which the samples were collected and the level of hygiene in the herds concerned [2].

The nested PCR was proven to be suitable for the detection of *H. pylori* in milk samples. In our further work on *H. pylori*, we will focus on modi-

fying the nested PCR protocol by adding dUTP to prevent contamination of the working environment with short fragments of amplified DNA [17, 18].

CONCLUSIONS

The study confirmed that the nested PCR is a highly sensitive method for the detection of *H. pylori* in raw milk samples. The high sensitivity of the method allows the detection of small numbers of bacterial cells in food. However, it is not possible to determine the viability of the examined bacteria. On the other hand, the method also allows the detection of bacteria that are viable but unculturable. The results of this study demonstrated the presence of *H. pylori* DNA in 47 % of samples of raw cows' and goats' milk originating from two selected farms in the Moravian region of the Czech Republic. This high percentage of *H. pylori* positive raw milk samples indicates a non-negligible risk to the consumer in terms of the potential zoonotic nature of *H. pylori* infection. A high risk of transmission of the infection may occur especially when raw milk is consumed without heat treatment.

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REFERENCES

1. Guessoum, M. – Guechi, Z. – Adnane, M.: First-time serological and molecular detection of *Helicobacter pylori* in milk from Algerian local-breed cows. Veterinary World, *11*, 2018, pp. 1326–1330. DOI: 10.14202/vetworld.2018.1326-1330.
2. Quaglia, N. C. – Dambrosio, A.: *Helicobacter pylori*: A foodborne pathogen? World Journal of Gastroenterology, *24*, 2018, pp. 3472–3487. DOI: dx.doi.org/10.3748/wjg.v24.i31.3472.
3. Velázquez, M. – Feirtag, J. M.: *Helicobacter pylori*: characteristics, pathogenicity, detection methods and mode of transmission implicating foods and water. International Journal of Food Microbiology, *53*, 1999, pp. 95–104. DOI: 10.1016/S0168-1605(99)00160-9.
4. Gomes, B. C. – De Martinis, E. C. P.: The significance of *Helicobacter pylori* in water, food and environmental samples. Food Control, *15*, 2004, pp. 397–403. DOI: 10.1016/S0956-7135(03)00106-3.
5. Qauglia, N. C. – Dambrosio, A. – Normanno, G. – Celano, G. V.: Evaluation of a nested-PCR assay based on the phosphoglucosamine mutase gene (*glmM*) for the detection of *Helicobacter pylori* from raw milk. Food Control, *20*, 2009, pp. 119–123. DOI: 10.1016/j.foodcont.2008.02.010.
6. Andersen, L. P. – Rasmussen, L.: *Helicobacter pylori* – coccoid forms and biofilm formation. FEMS Immunology and Medical Microbiology, *56*, 2009, pp. 112–115. DOI: 10.1111/j.1574-695X.2009.00556.x.
7. Bianchini, V. – Recordati, C. – Borella, L. – Gualdi, V. – Scanziani, E. – Selvatico, E. – Luini, M.: Helicobacteraceae in bulk tank milk of dairy herds from Northern Italy. BioMed Research International, *2015*, 2015, article ID 639521. DOI: 10.1155/2015/639521.
8. Dore, M. P. – Supulveda, A. R. – El-Zimaity, H. – Yamaoka, Y. – Osato, M. S. – Mototsugu, K. – Nieddu, A. M. – Realdi, G. – Graham, D. Y.: Isolation of *Helicobacter pylori* from sheep – implications for transmission to humans. American Journal of Gastroenterology, *96*, 2001, pp. 1396–1401. DOI: 10.1111/j.1572-0241.2001.03772.x.
9. Osman, E. Y. – El-Eragi, A. M. S. – Musa, A. M. – El-Magboul, S. B. – A/Rahman, M. B. – Abdo, A. E.: Detection of *Helicobacter pylori glmM* gene in bovine milk using nested polymerase chain reaction. Veterinary World, *8*, 2015, pp. 913–917. DOI: 10.14202/vetworld.2015.913-917.
10. Fujimura, S. – Kawamura, T. – Kato, S. – Tateno, H. – Watanabe, A.: Detection of *Helicobacter pylori* in cow's milk. Letters in Applied Microbiology, *35*, 2002, pp. 504–507. DOI: 10.1046/j.1472-765X.2002.01229.x.
11. Poms, R. E. – Tatini, S. R.: Survival of *Helicobacter pylori* in ready-to-eat foods at 4 °C. International Journal of Food Microbiology, *63*, 2001, pp. 281–286. DOI: 10.1016/s0168-1605(00)00441-4.
12. Quaglia, N. C. – Dambrosio, A. – Normanno, G. – Parisi, A. – Patrono, R. – Ranieri, G. – Rella, A. – Celano, G. V.: High occurrence of *Helicobacter pylori* in raw goat, sheep and cow milk inferred by *glmM* gene: A risk of food-borne infection? International Journal of Food Microbiology, *124*, 2008, pp. 43–47. DOI: 10.1016/j.ijfoodmicro.2008.02.011.
13. Talaei, R. – Souod, N. – Momtaz, H. – Dabiri, H.: Milk of livestock as a possible transmission route of *Helicobacter pylori* infection. Gastroenterology and Hepatology from Bed to Bench, *8*, 2015, pp. S30–S36. ISSN: 2008-2258.
14. Ho, S. – Hoyle, J. A. – Lewis, F. A. – Secker, A. D. – Cross, D. – Mapstone, N. P. – Dixon, M. F. – Wyatt, J. I. – Tompkins, D. S. –Taylor, G. R. – Quirke, P.: Direct polymerase chain reaction test for detection of *Helicobacter pylori* in humans and animals. Journal of Clinical Microbiology, *29*, 1991, pp. 2543–2549. DOI: 10.1128/jcm.29.11.2543-2549.1991.
15. Mousavi, S. – Dehkordi, F. – Rahimi, E.: Virulence factors and antibiotic resistance of *Helicobacter pylori* isolated from raw milk and unpasteurized dairy products in Iran. Journal of Venomous Animals and Toxins including Tropical Diseases, *20*, 2014, article 51. DOI: 10.1186/1678-9199-20-51.

16. Rahimi, E. – Kheirabadi, E. K.: Detection of *Helicobacter pylori* in bovine, buffalo, camel, ovine, and caprine milk in Iran. Foodborne Pathogens and Disease, *9*, 2012, pp. 453–456. DOI: 10.1089/fpd.2011.1060.
17. Herrera-Sandoval, C. A. – Lopera-Mesa, T. M.: Incorporación de uracilo ADN glicosilasa / dUTPs en la reacción de PCR anidada para detectar *Plasmodium falciparum* y *Plasmodium vivax*: una estrategia para reducir el riesgo de contaminación. (Uracil-DNA-glycosilase system (UDG-dUTPs) incorporation in nested PCR reaction for *Plasmodium falciparum* and *Plasmodium vivax* detection: a strategy for reducing risk of contamination. Actualidades Biológicas, *42*, 2020, pp. 1–12. DOI: 10.17533/udea.acbi.v42n113a06. In Spanish.
18. Hu, Y.: Regulatory concern of polymerase chain reaction (PCR) carryover contamination. In: Samadikuchaksaraei, A. (Ed.): Polymerase chain reaction for biomedical applications. London : IntechOpen, 2016. ISBN: 978-953-51-2796-3, 978-953-51-2795-6 (print), 978-953-51-4133-4 (e-book). DOI: 10.5772/66294.

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