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International Journal of Food Microbiology 63 (2001) 281–286

INTERNATIONAL JOURNAL OF Food Microbiology

www.elsevier.nl/locate/ijfoodmicro

Short communication

Survival of *Helicobacter pylori* in ready-to-eat foods at 4°C

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Received 11 March 2000; received in revised form 11 September 2000; accepted 29 September 2000

Abstract

The survival of *Helicobacter pylori* (NCTC 11638) in various semiprocessed and fresh, ready-to-eat foods, and one raw chicken was studied at 4°C and under aerobic conditions by experimentally inoculating these with 10^4 CFU. Cells were concentrated by two centrifugation cycles followed by plating onto selective blood agar medium made from Wilkins-Chalgren agar supplemented with 5% whole horse blood, and 30 mg/l colistin methanesulfonate, 100 mg/l cycloheximide, 30 mg/l nalidixic acid, 30 mg/l trimethoprim, and 10 mg/l vancomycin. *H. pylori* was recovered from spiked pasteurized milk and tofu samples up to 5 days and from spiked leaf lettuce and raw chicken up to 2 days. *H. pylori* could not be recovered from yogurt after any length of storage time. *H. pylori* is unlikely to grow in foods; however, it may survive in low acid–high moisture environments under refrigeration and pose a possible risk for transmission of infection via foods.

Keywords: *Helicobacter pylori*; Transmission; Detection method; Foods

1. Introduction

Helicobacter pylori is an emerging human pathogen. Since its discovery in 1982 by Marshall and Warren (landmark paper, 1984), *H. pylori* has become widely recognized as a major cause of gastritis and gastroduodenal ulceration (NIH Consensus Conference, 1994). Furthermore, *H. pylori* may play an important role in the development of adenocarcinoma and lymphoma of the stomach (Cover and Blaser, 1995). Other findings associate *H. pylori* infection also with cardiovascular disease (Ponzetto et al., 1996). The prevalence of *H. pylori* infection is worldwide and maybe as high as 80% in developing countries and up to 40% in developed countries (Cover and Blaser, 1995; Dubois, 1995). However, the way in which *H. pylori* infection is transmitted is poorly understood. Although current evidence suggests that the reservoir of infection is the human stomach, the mode of transmission is unclear.

H. pylori has been isolated from feces (Thomas et al., 1992) and it has also been detected in dental plaque by culture (Krajden et al., 1989; Majmudar et

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0168-1605/01/$ – see front matter
PII: S0168-1605(00)00441-4

al., 1990) and by polymerase chain reaction (PCR) (Banatvala et al., 1993), and in saliva (Ferguson et al., 1993). For etiological studies the presence or absence of *H. pylori* in the environment has to be elucidated. Due to difficulties in isolation of *H. pylori* from environmental sources only the use of sophisticated and expensive techniques such as filtration, immunoseparation, polymerase chain reaction (PCR) and hybridization with specific probes has been shown successful in detecting *H. pylori* from samples from other than clinical origin (Velazquez and Feirtag, 1999). *H. pylori* has not been successfully cultured from the environment; yet, it has been detected in drinking water in Peru by PCR (Hulten et al., 1996). Thus, the notion of fecal–oral or oral–oral transmission of *H. pylori* is supported in the literature (Cave, 1996). Although the currently disputed arguments for and against fecal–oral, oral–oral and gastric–oral spread have not been conclusive (Stone, 1999), most researchers agree that *H. pylori* infection is acquired by close contact with infected individuals in early childhood (Oderda, 1999; Miyaji et al., 2000). This does not, however, preclude the possibility of food, water, or pets as vehicles for transmission of *H. pylori*. In fact Goodman et al. (1996) suggested a multiple-pathway phenomenon rather than a single mode of transmission.

The objective of this investigation, which was undertaken between 1995 and 1997, was to evaluate selective conditions/methods for recovery of *H. pylori* from inoculated food samples to provide further means of evaluating the possible pathways of *H. pylori* transmission. The reservoir for *H. pylori* is the human gastric mucosa. As oral–oral or fecal–oral transmissions of *H. pylori* are very likely (Cave, 1996), the organism will have to survive in the external environment for a considerable period of time. Clustering of *H. pylori* infections in families also suggests that besides direct transmission from person to person an indirect way via 'food from the same table' so to speak is very possible. This work shows whether and how long *H. pylori* can survive in refrigerated foods.

2. Materials and methods

2.1. Bacterial strain

Helicobacter pylori (NCTC 11638) was obtained from the National Collection of Type Cultures (London, UK) and it was revived according to the manufacturer's instructions. *H. pylori* was further propagated in Brucella broth (Difco Laboratories, Detroit, MI, USA) supplemented with 5% defibrinated horse blood (Micropure Medical, White Bear Lake, MN, USA), which had been lysed by several freeze–thaw cycles in the laboratory. Throughout all experiments presumptively positive colonies of *H. pylori* were identified by carbol fuchsin staining, a catalase test and a rapid urease test (Christensen's broth). Identity of *H. pylori* (NCTC 11638) was confirmed by comparing RAPD fingerprinting patterns of the organism at the beginning and at the end of the experiments by employing a 16-mer primer with homology to a universally conserved region within the 16 rRNA gene (Poms, 1997).

2.2. Media

Tryptic soy agar (Difco) and Wilkins-Chalgren agar (Difco) supplemented with 5% whole defibrinated horse blood (Micropure Medical) served as non-selective media to enumerate colony forming units from the various foods tested.

Wilkins-Chalgren agar (Difco) supplemented with 5% whole defibrinated horse blood (Micropure Medical) containing 30 mg/l colistin methanesulfonate, 100 mg/l cycloheximide, 30 mg/l nalidixic acid, 30 mg/l trimethoprim and 10 mg/l vancomycin (all Sigma, St. Louis, MO, USA), was used for isolation of *H. pylori* (NCTC 11638).

Serial dilutions of foods (containing *H. pylori* and the naturally existing flora) were made in Tryptic Soy broth (Difco). TSB was also used as the stomaching diluent in the isolation procedure.

2.3. Incubation conditions

Helicobacter pylori requires microaerobic conditions and it grows optimally at 37°C. Plates and broth cultures were therefore incubated in a microaerobic environment created by CO_2 and H_2 generating Campy Pak Plus envelopes (BBL Becton-Dickinson Microbiology Systems, Cockeysville, MD, USA) in an anaerobic jar (BBL). To maintain a moist atmosphere, a moist paper towel was placed in the jar. Plates and broth cultures of *H. pylori* were incubated at 37°C for 5–7 days.

2.4. Foods

Prepacked hard Nigari Tofu stored in water, plain yogurt, pasteurized skim milk, fresh leaf lettuce, and prepacked boneless, skinless chicken thighs were obtained from a local grocery store and were used within 2 days.

2.4.1. Tofu, yogurt, lettuce and chicken

Tofu, yogurt, lettuce and chicken thighs were portioned and weighed individually (50 ± 0.5 g portions) into stomacher 400 bags (Seward, Medical, London, UK).These were closed with a wire tie and kept refrigerated at 4°C until used.

2.4.2. Milk and tofu storage water

Nine ml of milk and 9 ml of tofu water were each transferred to separate test tubes, and were also kept at 4°C.

2.5. Bacterial inoculation

For inoculation, a 5-day-old 37°C culture of *H. pylori* (NCTC 11638) was serially diluted in Tryptic Soy broth to obtain a concentration of ca. 6×10^4 CFU/ml. One ml of this cell suspension was added to the contents of each stomacher bag. Cells of *H. pylori* were submersed into the samples where possible, but remained on the surface of the chicken and the lettuce samples. Nine separate samples of each of tofu, yogurt, lettuce, and chicken were inoculated to provide one preparation of each kind of food for every sampling point. The stomacher bags were then sealed tight with a plastic-coated wire and stored at 4°C.

For skim milk and tofu storage water samples the 5-day-old culture of *H. pylori* (NCTC 11638) was serially diluted in skim milk and tofu water, respectively, to obtain a concentration of 6×10^4 CFU/ml. One ml of the diluted cell-milk-suspension was added to 9 ml of skim milk, and 1 ml of cell-tofu storage water-suspension to 9 ml tofu storage water, so that the final concentration of *H. pylori* for both samples was about 6×10^3 CFU/ml. Both preparations were stored in capped glass reaction tubes at 4°C.

2.6. Bacterial isolation

2.6.1. Tofu, lettuce, chicken

At sampling time 100 ml of TSB were added to each stomacher bag containing 50 ± 0.5 g sample. The stomacher bag plus contents was stomached for 2 min in a stomacher lab blender 400 (Seward, UAC House, London, UK). The stomached sample was then transferred to a sterile 250-ml centrifuge tube (Nalgene), and centrifuged at 4°C for 5 min at $2000\times g$ (Sorvall RC-5 Superspeed Refrigerated Centrifuge) to eliminate insoluble solids from the sample. The pellet was discarded and the supernatant was transferred into another sterile 250-ml centrifuge tube and centrifuged at 4°C for 30 min at $5000\times g$. This time, the supernatant was discarded and the pellet resuspended in 5 ml of TSB. Aliquots (0.1 ml) of this suspension were spread-plated on selective blood agar plates, in duplicate.

2.6.2. Yogurt

As yogurt was already semi-liquid only 10 ml of Tryptic Soy broth were added to 50 ± 0.5 g of yogurt sample contained in a stomacher bag and stomached for 2 min. Duplicate 0.1 ml volumes of the suspension were plated on selective *H. pylori* blood agar plates.

2.6.3. Skim milk, tofu storage water

Aliquots (0.1 ml) of each of the experimentally inoculated skim milk and tofu storage water were directly plated onto selective *H. pylori* blood agar plates, in duplicate.

2.7. Sampling

Samples were examined at the beginning of the experiment, and after 1, 2, 3, 4, 5, 7, 10, and 14 days of storage at 4°C. At every sampling point one 50 ± 0.5 g sample of each of the inoculated foods contained in separate stomacher bags was processed as described above, and the 0.1 ml aliquots of the obtained cell suspensions as well as the 0.1 ml aliquots of the liquid samples were plated on selective blood agar plates and incubated under the conditions described above.

In addition, non-inoculated controls of each of the investigated foods were examined at the beginning of the experiment. Therefore, original samples were prepared as described above, but without inoculation with *H. pylori*. Serial dilutions of each sample's cell suspension were made, and 0.1 ml aliquots were plated in duplicates on non-selective Tryptic Soy agar (incubated under aerobic conditions at 37°C),

and on non-selective Wilkins-Chalgren blood agar and selective Wilkins-Chalgren blood agar plates (both incubated under microaerobic conditions at 37°C). This was done to evaluate recovery of the natural flora of the various foods, and to determine whether *H. pylori* was already present in any of the foods tested.

3. Results and discussion

The microbial level and the pH of the various foods used in this study are shown in Table 1. Tryptic Soy agar plates, which were incubated for 48 h at 37°C under aerobic conditions, yielded slightly higher total counts of all organisms for milk, tofu storage water, lettuce, and chicken than the blood agar plates, which were incubated under microaerobic conditions at 37°C for 72 h. Yogurt showed low numbers of aerobic organisms, but a very high concentration of microaerophilic organisms (lactic acid bacteria). Tofu did not yield any colonies on either plate agar system.

Wilkins-Chalgren blood agar supplemented with 30 mg/l colistin methanesulfonate, 100 mg/l cycloheximide, 30 mg/l nalidixic acid, 30 mg/l trimethoprim and 10 mg/l vancomycin was found to be highly selective for *H. pylori*.

In this present study *H. pylori* was not isolated from any of the food systems before experimental inoculation. From spiked skim milk and tofu storage water samples culturable cells of *H. pylori* (NCTC 11638) could be recovered up to 5 days but not after 7 days of storage at 4°C (see Fig. 1). There was a decrease of about 1 log after 4 days. After day 5 there was a rapid decline over more than two logs to below the level of sensitivity (< 10 CFU/ml)

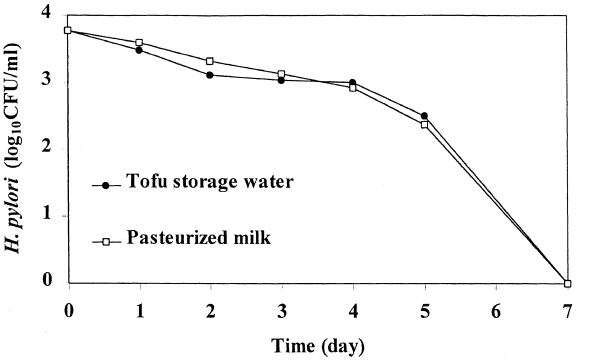

Fig. 1. Survival of *H. pylori* in refrigerated (4°C) pasteurized skim milk and tofu storage water.

H. pylori could only be recovered from lettuce and raw chicken for up to 2 days after inoculation (see Fig. 2). The reason for this rapid loss of detectability could be the lack of protection against oxygen and desiccation on the surface of the lettuce and the chicken as opposed to the protecting liquid phase of tofu, tofu storage water, and milk. Furthermore, lettuce and raw chicken with ca. 6.2×10^5 CFU/g and ca. 7.0×10^5 CFU/g, respectively, show a dense population of their natural bacterial flora. Due to its slow growth and sensitivity to physical and chemical stress, *H. pylori* has a great disadvantage in the competition for nutrients and resistance to metabolites synthesized by commensals, which might also explain this rapid decrease in recoverable *H. pylori*.

Compared to the other foods examined there was a rapid decrease of culturable *H. pylori* in yogurt immediately after inoculation and none could be

Table 1
Microbial load and pH of food systems used for the study on the survival of *H. pylori*

Food	pH	Aerobic[a] (CFU)	Microaerophilic[b] (CFU)
Lettuce	6.2	6.2×10^5/g	3.8×10^5/g
Tofu	6.0	< 10/50 g	< 10/50 g
Tofu storage water	6.5	500/ml	400/ml
Pasteurized milk	6.8	2.5×10^3/ml	2.3×10^3/ml
Yogurt	4.0	5/g	3.0×10^7/g
Chicken	6.8	7.0×10^5/g	3.5×10^5/g

[a] Plated on Tryptic Soy agar.
[b] Plated on Wilkins-Chalgren blood agar and incubated under microaerobic conditions (reduced oxygen, increased CO_2 and H_2).

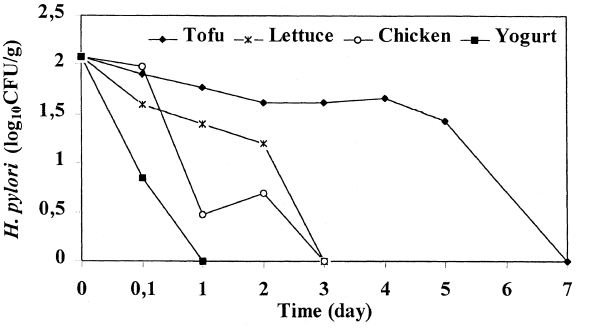

Fig. 2. Survival of *H. pylori* in refrigerated (4°C) solid and semi-solid food systems.

recovered after 1 day (see Fig. 2). According to the findings of West et al. (1992) that postulated that *H. pylori* (NCTC 11637) did not survive in acetate buffer at any pH between 3.5 and 5.0 and to Midolo et al. (1995), who stated that *H. pylori* (NCTC 11637) was inhibited by organic acids from lactic acid bacterial growth, *H. pylori* would not be expected to survive in an organic acid environment provided by lactic acid bacteria in yogurt fermentations.

Pasteurized skim milk, tofu, yogurt and lettuce are ready-to-eat products, which, when handled by humans, could serve as a potential source of infection for *H. pylori*. Although heat treatment would kill *H. pylori* (pasteurization of milk, preparation of tofu), the possibility of secondary contamination remains. As oral–oral or fecal–oral transmissions of *H. pylori* are very likely, the organism will have to survive in the external environment for a considerable period of time. This work shows that skim milk, tofu, tofu storage water, and lettuce can act as vehicles for *H. pylori* transmission.

The isolation procedure used in this work allowed isolation of *H. pylori* from various foods, but it lacked sufficient sensitivity to reproducibly recover very low numbers of *H. pylori* (detection limits: about 10 CFU per ml liquid sample and 50 CFU per 50 g solid matter to recover at least one colony per plate). However, the possible change of the culturable into the viable but non-culturable (VNC) state of *H. pylori* was not investigated in this study.

Further research is required to develop isolation procedures from environmental samples in order to learn more about the mode of transmission of *H. pylori* infection.

Acknowledgements

Funding for this project was provided by the Minnesota–South Dakota Dairy Foods Research Center and a grant from the Austrian Department of Science and Research. Our sincere thanks go to Dr. Mueller and Dr. Kneifel, both from the University of Agricultural Sciences in Vienna, Austria, who made this international collaboration possible. Further we would like to thank Dr. O'Sullivan from the University of Minnesota for providing primer information and technical assistance in the RAPD-PCR.

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