Microb Health Dis 2020; 2: e306

HELICOBACTER PYLORI INFECTION IN SHEPHERDS, SHEEP AND SHEEP-DOGS

M. P. Dore[1,4], G. M. Pes[1], G. Sanna[2], A. R. Sepulveda[3], D. Y. Graham[4]

[1]Dipartimento di Scienze Mediche, Chirurgiche e Sperimentali, University of Sassari, Sassari, Italy
[2]Servizio di Sanità Animale, Area Socio Sanitaria Locale di Olbia, Olbia, Italy
[3]Department of Pathology, The George Washington University, Washington DC, USA
[4]Department of Medicine, Michael E. DeBakey VA Medical Center and Baylor College of Medicine, Houston, TX, USA

Antonia Rogado Sepulveda and Yates Graham are equal contributors

Corresponding Author: Maria Pina Dore, MD, Ph.D; e-mail: mpdore@uniss.it

Abstract – ***Objectives:*** Previous studies demonstrated a high prevalence of *Helicobacter pylori* infection among shepherds, sheep, sheep raw milk and children having contact with sheep, suggesting a model of transmission of infection at elevated frequency. The aim of this study was to examine whether *H. pylori* infection may be present in sheep-dogs.

Material and Methods: Stool, serum, and wool were collected from sheep and sheep-dogs belonging to different flocks chosen because the shepherds had documented (^{13}C-UBT) *H. pylori* infection. *H. pylori* status in the animals was evaluated using a homemade anti-*H. pylori* IgG enzyme-linked immunosorbent assay (ELISA) for sheep and sheep-dogs. Stool samples from sheep and sheep-dogs were also analyzed using the stool antigen test (HpSA) developed for humans. Results were expressed as optical density (OD_{450}). PCR amplification was performed using primers specific for *H. pylori* [*cagA*, *vacA* (s and m region), *iceA*, *babA2*] as well as primers specific for *Helicobacter* species (16S rRNA) from the DNA extracted from sheep wool wash surrounding the mammalian glands.

Results: 58 animals were studied (44 sheep, 8 lambs and 6 sheep-dogs). The *H. pylori* antigen test was positive in 82% (36/44) and in 100% of sheep and sheep-dogs stool samples, respectively. Lamb stool samples were weakly positive. High anti *H. pylori* IgG serum levels were detected in all 6 sheep-dogs and in 42 of 44 sheep. Genomic *H. pylori* and *Helicobacter* species DNA was not detected from any of the sheep wool samples collected.

Conclusions: Although cross-reactivity cannot be excluded, the presence of the *H. pylori* antigen in stools obtained from sheep and from sheep-dogs, and the strong positivity for anti *H. pylori* IgG in both coupled with the ability to isolate *H. pylori* from sheep milk and stomachs, suggest that interaction between species may enhance the spread of bacteria in heavily contaminated settings.

Keywords: *Helicobacter pylori* infection, Sheep, Sheep-dogs.

INTRODUCTION

Helicobacter pylori infection is invariably related to inflammation and has been linked to benign, premalignant, and malignant lesions of the digestive system, including chronic active gastritis, peptic ulcers, atrophic gastritis, intestinal metaplasia, gastric adenocarcinomas and lymphomas of mucosa-associated lymphoid tissue[1].

Humans are the primary reservoir and the most common modes of transmission are oral-oral and fecal-oral. Most infections are acquired early in life probably through close contacts with parents, especially the mother and from other children[2]. In developing countries, the transmission *via* contaminated food or water might be a significant mechanism of primary infection in children and of reinfection among adults[3]. Differences among various populations and among ethnic groups are likely related to differences in cultural practices as they relate to hygiene and sanitation[4].

Beside the established reservoir of the human stomach, several animals, mostly living in the human environment, have been reported to harbor *H. pylori* in their stomachs[5]. In addition, an indirect evidence for a zoonotic source comes for studies[6-8] showing a higher prevalence of antibodies against *H. pylori* in abattoir workers, such as butchers, slaughterers, and veterinarians. In previous studies, we suggested that sheep might also be a reservoir, at least in Sardinia, Italy. For example, we observed a very high prevalence of *H. pylori* infection in Sardinian shepherds[9]. The prevalence among shepherds was approximately 100% and was significantly higher than among household members with no direct animal contact. Similarly, in Polish children with sheep contact a prevalence of *H. pylori* significantly higher than those from urban area was detected[10]. Moreover, *Helicobacter* genus-specific 16S rRNA and *H. pylori vacA* gene DNA was amplified from raw sheep milk samples collected from different flocks in Northern Sardinia[11]. Further, we were able to isolate *H. pylori* from raw sheep milk and the amplified genotype was *vacA s1b-m2*, *iceA2* allele positive, *babA2*-negative, and *cagA*-positive. The 5' region positive, middle conservative region-positive and the 3' repeat regions of *cagA* were of the type specific for Western countries (Italian type)[11,12]. Additional evidence demonstrated the presence of *H. pylori* in the stomach of sheep[10,12], suggesting these ruminants as a possible ring in the epidemiological model on the transmission chain of *H. pylori* infection, at least in certain circumstances, such as sheep-rearing. For example, in Sardinia the prevalence of *Echinococcus* disease was historically one of the highest in the Mediterranean basin and still remains particularly high in areas where pastoralism is the main economic activity[13,14]. Accordingly, across countries, the highest prevalence (8.7%) was registered among Mongolian and Kazak pastoralist communities[15].

The life cycle of *Echinococcus* tapeworm includes a definitive host, usually sheep-dogs, an intermediate host such as sheep, and human beings as incidental hosts. Because the transmission of *Echinococcus* eggs is closely related with the practice of sheep-rearing, direct interactions between shepherds, sheep and sheep-dogs are important in the diffusion of the zoonosis[16].

All these observations prompted us to confirm with further investigations (stool, serum, wool) the presence of *H. pylori* in sheep, lambs and sheep-dogs.

MATERIALS AND METHODS

Sample Collection

All samples were obtained from randomly selected sheep and sheep-dogs belonging to five different flocks located in the same geographical area of Northern Sardinia. The shepherds of the chosen flocks were previously identified as positive for *H. pylori* infection by ^{13}C urea breath test. From each animal studied a stool sample was collected and stored at -20°C until assayed. Stool specimens were obtained directly from the rectum in aseptic conditions. In addition, the wool surrounding the mammary glands from adult sheep was collected and immersed into 50 mL of Brucella broth and stored at -80°C until used for molecular studies. In addition, blood samples were obtained from each animal studied. Blood was centrifuged and the serum frozen at -20°C until processed. All sampling was done by a trained veterinarian (G.S.).

H. pylori Culture

Selective brain heart infusion (BHI)-agar plates, (Difco Laboratories, Detroit, MI, USA) containing 7% defibrinated horse blood (BHIB-agar plates; Cocalico Biologicals, Reamstown, PA, USA) and vancomycin (Sigma-Aldrich, St Louis, MO, USA), nalidixic acid (Sigma-Aldrich, St Louis, MO, USA), amphotericin B (Sigma-Aldrich, St Louis, MO, USA), trimethoprim (Sigma-Aldrich, St Louis, MO, USA), were inoculated with 100 μL of Brucella broth containing the wool surrounding the mammalian glands of adult sheep. Plates were incubated at 37°C under 12% CO_2 and 95-99% relative humidity and observed daily for up to 3 weeks for *H. pylori*-like colony growth.

ELISA Testing

Sheep and dog sera were evaluated for the presence of IgG antibodies against *H. pylori* by a homemade indirect enzyme-linked immunosorbent assay (ELISA). A crude sonicate extract from a pool of 15 *H. pylori* isolates was used as antigen. Briefly, 96-well microtiter plates (Nunc, Denmark) were coated with the antigen by incubating 100 µL of a 20-µg/mL antigen solution in 0.1 mol/L sodium carbonate buffer at pH 9.6 and kept overnight at room temperature. After washing in PBS, nonspecific sites were blocked with 200 µL of buffer (1% BSA in PBS) for 2 h. Blocking buffer was removed, and 100 µL of serum sample, diluted 1:200 in blocking buffer was incubated in duplicate for 1 h at room temperature. After three washes, the wells were incubated with 100 µL of horseradish peroxidase-conjugated rabbit Ig anti-sheep IgG and rabbit Ig anti-dog IgG (Dako, Spa, Milano, Italy), respectively, diluted 1:2000 for 30 min. All incubations were performed at room temperature. After four washes, the bounding antibodies were detected with H_2O_2-tetramethylbenzidine (TMB) solution and A_{450} was quantified with a Molecular Devices Corp. (San Jose, CA, USA) V_{max} plate reader. The samples were run in duplicate. Lamb serum samples known as negative served as control[12]. On the base of previous results[12], ELISA readings under a cut-off value of 0.400 OD were considered negative.

HpSA

The HpSA ELISA (Premier Platinum HpSA, Meridian Diagnostics Inc., Cincinnati, OH, USA) was performed according to manufacturer's recommendations. After addition of a stopping solution, the results were read by spectrophotometry at 450/620 nm double wavelength. According to the manufacturer's guidelines, an OD <0.100 was defined as negative, between 0.100-0.120 uncertain, and >0.120 surely positive.

Molecular Studies

Genomic DNA was extracted from Brucella broth containing sheep wool. The Brucella broth in which the wool was suspended was put into new bottles and the wool was washed three times with 50 mL of PBS which was added to the original broth. Initially the broth was brown due to contamination by milk, stool, and dirt. The original broth and the wash fluids were centrifuged at 12000 rpm for 10 min and the pellets were used for DNA extraction using a commercially available kit (QIAamp Tissue kit; QIAGEN, Inc., Chatsworth, CA, USA). Polymerase chain reaction (PCR) was performed using primers specific for *H. pylori* [*cagA, vacA* (s and m region), *iceA, babA2*] and for *Helicobacter* species (16S rRNA) as previously described[11,17].

RESULTS

A total of 58 animals were studied (44 sheep, 8 lambs and 6 sheep-dogs). The sheep ranged in age from five months to 5-year-old. There were 19 adult sheep (> 3 years). The lambs ranged in age from 0 to 4-month-old. The majority (95% or 42/44) of sheep serum samples contained IgG that recognized the crude *H. pylori* antigens (Figure 1). IgG against *H. pylori* were not detected in six 4-month-old lambs, whereas they were present in the 2-day old lambs probably representing immunoglobulins transmitted from the dams (Figure 1). All six sheep-dogs demonstrated IgG against *H. pylori* (Figure 1).

The results of the HpSA stool antigen test were positive in 82% (36/44), indeterminate in 11.4% (5/44) and negative in 2.3% (1/44) stool samples from sheep. The age of the sheep had no significant effect on *H. pylori* prevalence. Stool samples from lambs were typically in the gray zone. Stool samples from all 6 sheep-dogs were positive.

No *H. pylori* organism from any wool samples stored in Brucella broth were cultured. A total of 8 wool wash sheep samples were used for PCR. No PCR products were obtained from DNA using primers specific for *H. pylori* (*cagA, vacA, iceA, babA2*) or with primers specific for *Helicobacter* species (16S rRNA).

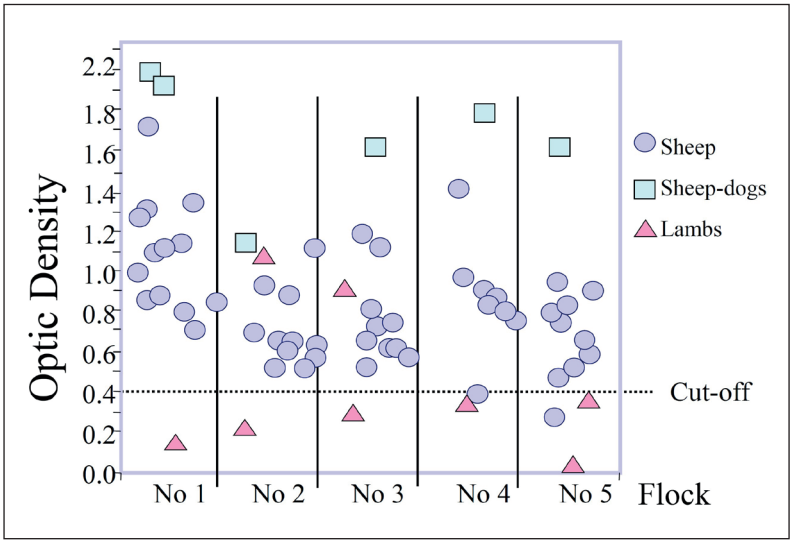

Figure 1. Values of the optical density of the serum in the sheep (circles), in the lambs (triangles) and in the sheep dogs (squares) collected from five different flocks. The line marks the cut-off at OD 0.4.

DISCUSSION

In this study we confirmed the presence of *H. pylori* antigen in stools obtained from sheep and from sheep-dogs. In addition, a strong positivity for anti *H. pylori* IgG was detected in both animals and an active infection in the attending shepherds suggesting a vicious circle in the transmission of the infection composed by different rings.

We previously reported that the prevalence of *H. pylori* infection in Sardinian asymptomatic shepherds was closely associated with direct contact with sheep and sheep-dogs[9], and the prevalence remains, even now, very high, especially in shepherds still practicing manual milking process (almost 100%, personal observation). Similarly, in a study conducted in Polish shepherds, a prevalence of *H. pylori* infection by ^{13}C-UBT of 97.6% and of 86% was detected[18]. In subjects without direct contact with sheep the prevalence was significantly lower (65.1%). Accordingly, in children from families living in Polish Tatra Mountains and having contact with sheep, *H. pylori* infection was significantly higher compared with age- and gender-matched urban controls[10]. In a previous study[12] we were able to culture *H. pylori* from smears collected from the *abomasum* mucosa of sheep belonging to flocks reared by shepherds identified to be positive for *H. pylori* infection. Infection was confirmed as *H. pylori* on the basis of colony morphology, biochemical reactions, Gram stain and genomic sequence analysis[12]. Similarly, in a total of 1,150 gastric specimens randomly collected from humans, cows, sheep and goats Ranjbar et al[19], were able to isolate the bacteria from 78% of adult patients and 70% of sheep. The results were confirmed using molecular analysis. Interestingly, in this study, *H. pylori* isolates from sheep showed a very high rate of antibiotic resistance against antimicrobials used for bacteria eradication in clinical practice for humans[19]. Moreover, sheep from Iran were found to be positive for *H. pylori* with a prevalent *vacA s1a/m1a* genotype and homology between samples from sheep and human ranging from 92.9% to 98.5%[20]. In the same geographic area, a considerable association was found between *s1b* allele from human gastric tissue and sheep raw milk[21]. However, in our study the wool collected from the sheep mammalian glands did not appear contaminated by *H. pylori*. It has been repeatedly reported that *H. pylori* is able to survive in dairy food[22]. Especially milk may act as a possible vector for the transmission of the bacteria in different circumstances[21,23]. According to that, we were able to culture the bacteria from raw sheep milk samples[11], and confirmed as *H. pylori* through molecular analysis by PCR with *Helicobacter* genus-specific rRNA and *vacA* gene subtype products amplification. The presence of *H. pylori* in milk from clinically healthy ovines was also confirmed by Rahimi et al[24] through culture and molecular methods. Furthermore, *H. pylori* strains were cultured from stool and milk samples obtained from sheep previously resulting positive for the bacteria presence by nested PCR and genotyping[25]. More interestingly, the authors demonstrated that laboratory mice fed with ultra-high temperature (UHT) milk, artificially contaminated with isolates previously cultured, showed high numbers of *H.*

pylori in the gastric mucosa[25]. In this study the genotypes of *H. pylori* strains isolated from stool and milk obtained from the same animal were identical suggesting a fecal contamination of milk[25].

Among animals, dogs have also been repeatedly demonstrated to be able to harbor *H. pylori* infection in their stomach[26,27], suggesting a role of this animal in the transmission of infection. In Sardinia, a region where sheep-rearing is highly diffuse, lives the largest community of sheep-dogs with a ratio of dogs to sheep of 1:20. In a previous sero-epidemiological study among approximately 95% of the pediatric population living in Northern Sardinia, it was found that children from rural villages had a significantly higher prevalence of *H. pylori* infection compared to those living in the adjoining urban area (35% *vs.* 13%; $p<0.005$)[28]. The variable that most closely associated with *H. pylori* infection was the presence of dogs in the home. In rural areas of Sardinia, most dogs are sheep-dogs. In the present study all sheep-dogs demonstrated IgG against *H. pylori*.

CONCLUSIONS

Although cross-reactivity could not be excluded, the presence of *H. pylori* antigen in stools obtained from sheep and from sheep-dogs and the high positivity for anti *H. pylori* IgG strongly suggest that interaction between species may enhance the spread of the bacteria, in heavily contaminated settings. This underlines the need to interdict some traditional practices, including clandestine butchery, drinking raw sheep milk or manual milking process in order to prevent or reduce transmission of pathogens in the communities of sheep-rearing attenders.

Conflict of Interest

Dr. Dore has received unrestricted grants from BioGaia (Stockholm, Sweden) in relation to probiotic therapy for *H. pylori* infection. Dr. Dore and Dr. Pes have received a grant from the *Regione Sardegna* No. 2014/2020, RASSR38231. Dr. Graham is a consultant for RedHill Biopharma regarding novel *H. pylori* therapies. He has received research support for culture of *Helicobacter pylori* and is the PI of an international study of the use of antimycobacterial therapy for Crohn's disease. He is also a consultant for BioGaia in relation to probiotic therapy for *H. pylori* infection and for Takeda in relation to *H. pylori* therapies. Dr. Sepulveda declares no potential conflicts of interest.

REFERENCES

1. Sugano K, Tack J, Kuipers EJ, Graham DY, el-Omar EM, Miura S, Haruma K, Asaka M, Uemura N, Malfertheiner P. Kyoto global consensus report on Helicobacter pylori gastritis. Gut 2015; 64: 1353-1367.
2. Malaty HM, Kumagai T, Tanaka E, Ota H, Kiyosawa K, Graham DY, Katsuyama T. Evidence from a nine-year birth cohort study in Japan of transmission pathways of Helicobacter pylori infection. J Clin Microbiol 2000; 38: 1971-1973.
3. Hulten K, Han SW, Enroth H, Klein PD, Opekun AR, Gilman RH, Evans DG, Engstrand L, Graham DY, El-Zaatari FA. Helicobacter pylori in the drinking water in Peru. Gastroenterology 1996; 110: 1031-1035.
4. Kikuchi S, Dore MP. Epidemiology of Helicobacter pylori Infection. Helicobacter 2005; 10 Suppl 1: 1-4.
5. Fox JG. Non-human reservoirs of Helicobacter pylori. Aliment Pharmacol Ther 1995; 9 Suppl 2: 93-103.
6. Vaira D, D'Anastasio C, Holton J, Dowsett JF, Londei M, Bertoni F, Beltrandi E, Grauenfels P, Salmon PR, Gandolfi L. Campylobacter pylori in abattoir workers: is it a zoonosis? Lancet 1988; 2: 725-726.
7. Morris A, Nicholson G, Lloyd G, Haines D, Rogers A, Taylor D. Seroepidemiology of Campylobacter pyloridis. NZ Med J 1986; 99: 657-659.
8. Husson MO, Vincent P, Grabiaud MH, Furon D, Leclerc H. Anti-Helicobacter pylori IgG levels in abattoir workers. Gastroenterol Clin Biol 1991; 15: 723-726.
9. Dore MP, Bilotta M, Vaira D, Manca A, Massarelli G, Leandro G, Atzei A, Pisanu G, Graham DY, Realdi G. High prevalence of Helicobacter pylori infection in sheperds. Dig Dis Sci 1999; 44: 1161-1164.
10. Plonka M, Bielanski W, Konturek SJ, Targosz A, Sliwowski Z, Dobrzanska M, Kaminska A, Sito E, Konturek PC, Brzozowski T. Helicobacter pylori infection and serum gastrin, ghrelin and leptin in children of Polish shepherds. Dig Liver Dis 2006; 38: 91-97.
11. Dore MP, Sepulveda AR, Osato MS, Realdi G, Graham DY. Helicobacter pylori in sheep milk. Lancet 1999; 354: 132.
12. Dore MP, Sepulveda AR, El-Zimaity H, Yamaoka Y, Osato MS, Mototsugu K, Nieddu AM, Realdi G, Graham DY. Isolation of Helicobacter pylori from sheep – implications for transmission to humans. Am J Gastroenterol 2001; 96: 1396-1401.

13. Conchedda M, Seu V, Capra S, Caredda A, Pani SP, Lochi PG, Collu C, Mura A, Gabriele F. Cystic echinococcosis in sheep in Sardinia: changing pattern and present status. Acta Trop 2012; 122: 52-58.
14. Conchedda M, Antonelli A, Caddori A, Gabriele F. A retrospective analysis of human cystic echinococcosis in Sardinia (Italy), an endemic Mediterranean region, from 2001 to 2005. Parasitol Int 2010; 59: 454-459.
15. Wang GZ, Feng XH, Chu XD, Er XD, Ar MN, Wen H. Epidemiological study on human echinococcosis in Hobuksar Mongolian autonomous county of Xinjiang. Chin J Endemiol 2009; 28: 214-217.
16. Gabriele F, Bortoletti G, Conchedda M, Palmas C, Ecca AR. Epidemiology of hydatid disease in the Mediterranean basin with special reference to Italy. Parassitologia 1997; 39: 47-52.
17. Yamaoka Y, Kodama T, Kashima K, Graham DY. Antibody against Helicobacter pylori CagA and VacA and the risk for gastric cancer. J Clin Pathol 1999; 52: 215-218.
18. Papiez D, Konturek PC, Bielanski W, Plonka M, Dobrzanska M, Kaminska A, Szczyrk U, Bochenek A, Wierzchos E. Prevalence of Helicobacter pylori infection in Polish shepherds and their families. Dig Liver Dis 2003; 35: 10-15.
19. Ranjbar R, Chehelgerdi M. Genotyping and antibiotic resistance properties of Helicobacter pylori strains isolated from human and animal gastric biopsies. Infect Drug Resist 2018; 11: 2545-2554.
20. Momtaz H, Dabiri H, Souod N, Gholami M. Study of Helicobacter pylori genotype status in cows, sheep, goats and human beings. BMC Gastroenterol 2014; 14: 61.
21. Talaei R, Souod N, Momtaz H, Dabiri H. Milk of livestock as a possible transmission route of Helicobacter pylori infection .Gastroenterol Hepatol Bed Bench 2015; 8(Suppl 1): 30-36.
22. Quaglia NC, Dambrosio A, Normanno G, Parisi A, Patrono R, Ranieri G, Rella A, Celano GV. High occurrence of Helicobacter pylori in raw goat, sheep and cow milk inferred by glmM gene: a risk of food-borne infection? Int J Food Microbiol 2008; 124: 43-47.
23. Turutoglu H, Mudul S. Investigation of Helicobacter pylori in raw sheep milk samples. J Vet Med B Infect Dis Vet Public Health 2002; 49: 308-309.
24. Rahimi E, Kheirabadi EK. Detection of Helicobacter pylori in bovine, buffalo, camel, ovine, and caprine milk in Iran. Foodborne Pathog Dis 2012; 9: 453-456.
25. Elhariri M, Hamza D, Elhelw R, Hamza E. Occurrence of cagA+ vacA s1am1i1 Helicobacter pylori in farm animals in Egypt and ability to survive in experimentally contaminated UHT milk. Sci Rep 2018; 8: 14260.
26. Chung TH, Kim HD, Lee YS, Hwang CY. Determination of the prevalence of Helicobacter heilmannii-like organisms type 2 (HHLO-2) infection in humans and dogs using non-invasive genus/species-specifc PCR in Korea. J Vet Med Sci 2014; 76; 73-79.
27. Hamza D, Elhelw R, Elhariri M, Ragab E. Genotyping and antimicrobial resistance patterns of Helicobacter pylori in humans and dogs associated with A2142G and A2143G point mutations in clarithromycin resistance. Microb Pathog 2018; 123: 330-338.
28. Dore MP, Malaty HM, Graham DY, Fanciulli G, Delitala G, Realdi G. Risk Factors Associated with Helicobacter pylori Infection among Children in a Defined Geographic Area. Clin Infect Dis 2002; 35: 240-245.