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***Helicobacter pylori* and Liver – Detection of Bacteria in Liver Tissue from Patients with Hepatocellular Carcinoma Using Laser Capture Microdissection Technique (LCM)**

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1. Introduction

Hepatocellular carcinoma (HCC) is the most common primary tumor of the liver in humans [1]. It is the fifth most common cancer in men (523,000 cases per year, 7.9% of all cancers) and the seventh among women (226,000 cases per year, 3.7% of all cancers) [2], with over a half of million new cases diagnosed annually. It is the second leading cause of the cancer related mortality in the world [3,4,5] and its prevalence differs according to geographic location, gender, age and ethnicity [6].

In general, the distribution of HCC cases presents great geographic variation, with higher incidence in Easter Asia and sub-Saharan Africa where infection with hepatitis B virus (HBV) is endemic with rates of over 20 per 100,000 individuals. Mediterranean countries such as Italy, Spain and Greece have intermediate rates of 10 to 20 per 100,000 individuals. The North and South America have a relatively low incidence (< 5 per 100,000 individuals) [4], although a rising incidence was observed in the USA, probably associated with the rise in hepatitis C virus (HCV) infection. Recent decreases in the incidence of HCC were reported among Chinese populations in Hong Kong, Shangai and Singapore; the incidence in Japan is also decreasing [3].

In Brazil, there are few reports on the prevalence of HCC [7]. It was suggested that the prevalence was considered low according to epidemiologic and retrospective studies [8]. In

1997, Brazilian researchers showed that HVB was the most common cause of liver disease in patients with HCC [9]. After that, another survey demonstrated that in Southeastern and Southern Brazil, HCV accounted for over 55% of cases. In the Northeast and North, HCV accounted for less than 50% and HBV accounted for 22-25% of cases; hepatitis B was more prevalent in the Northern than in the Southern regions [10].

The development of HCC has been attributed to several risk factors. In general, chronic viral infection with HBV and HCV is considered the major cause of HCC in 75-80% of cases [1], although HBV is globally considered the leading risk factor responsible for 50% of cases [11]. Furthermore, cirrhosis [12], exposure to the carcinogenic fungal aflatoxin B1 [13], inherited diseases [14], Wilson's disease [15] and heavy alcohol consumption [16] are also risk factors attributed to its development. Recently, upcoming risk factors for HCC include obesity, diabetes and related nonalcoholic fatty liver disease [3].

In 1994, researchers described the infectious agent *Helicobacter (H.) hepaticus* and its role in causing active hepatitis and associated liver tumors in mice [17]. Since then, several studies related to *H. hepaticus* experimental infection have demonstrated that this bacterium may induce a strong inflammatory change in the liver leading to HCC. Considering that *H. pylori* was classified as a class I carcinogen [18] and *Helicobacter* spp. DNA was detected in hepatic tissue from patients with different hepatobiliary diseases, it has been proposed that in humans, as in animals, *Helicobacter* spp. may also colonize and induce chronic hepatic diseases mainly HCC.

In fact, studies related to the possible association between *H. pylori* and hepatobiliary diseases have been developed since 1998, when *Helicobacter* DNA was identified in Chilean patients with chronic cholecystitis [19]. After that, a variety of researches have been conducted to verify the role of *H. pylori* in the development of HCC [20, 21, 22, 23, 24, 25, 26 e 27]. Considering the role of chronic inflammation and infection in the development of cancer, in the case of HCC, future studies should be performed to verify the role of *Helicobacter* infection in the liver pathophysiology [28]. However, whether this bacterium causes liver tumor or acts as a cofactor in the process of carcinogenesis needs to be confirmed.

The mechanism by which *H. pylori* colonizes the human liver is not totally enlightened. The *H. pylori* DNA detected in the liver tissue may result from bacterial translocation from the stomach into the blood through the portal system, especially in the later stages of chronic liver disease when portal hypertension occurs [1, 29, 30]. In addition, the bacteria may reach the liver by phagocytes and macrophages or circulating retrograde transfer from the duodenum [31]. However, the studies involving the growth of *H. pylori* from the HCC liver reinforce the bacterial colonization ruling out the possibility of retrograde contamination [32, 33]. Additionally, no other bacteria from the digestive tract are associated with human hepatocarcinogenesis [23, 34].

Several researchers have suggested that *H. pylori* may damage hepatocytes in vitro by a cytopathic effect in a liver and HCC cell lines [35, 36]. Furthermore, it was demonstrated in a HCC cell line (Huh7) that an inoculum of *H. pylori* was able to adhere and internalize into hepatocytes and this result was also dependent on virulence factors of bacteria [37]. Proteomic

analysis of human hepatic cell line (HepG2) co-cultured with *H. pylori* have revealed that bacteria may exert the pathological effect on HepG2 cells by up-regulating the expression of some proteins enrolled in transcription regulation, signal transduction and metabolism [38].

In most pathology laboratories, archives of formalin-fixed paraffin-embedded (FFPE) tissues represent the only tissue specimens available for routine diagnostics. A major advantage of such archives is that long-term clinical data is often available [39]. Furthermore, another benefit of using FFPE tissues is that they are the easiest to store and transport [40]. Because of this, FFPE tissues have been used in PCR-based studies related to cancer research, genetics, infectious diseases and molecular epidemiology [41]. Additionally, the use of FFPE tissue also allows employing modern transcriptomic and epigenomic methods with nucleic acids [39].

However, isolating high-quality genomic DNA from FFPE sections remains a challenge for researchers. Formalin is the most commonly used tissue fixative worldwide because it offers the best compromise between cost, practicality and morphological fixative properties [39, 42]. However, the fixation of tissue in formalin leads to extensive protein-DNA cross-linking of all tissue components and nucleic acids isolated from these specimens are highly fragmented [43]. This is particularly troublesome when long DNA regions are amplified, old paraffin blocks are used or fixation time is over three days [41, 44]. Because of this, FFPE tissue requires special protocols in order to extract small amounts of DNA suitable for amplification [45]. Nevertheless, methods of DNA extraction from FFPE tissue are generally laborious and time consuming.

Although studies on the role of the *H. pylori* in the development of HCC were more frequent in the last decade [46], most of them presents a prospective nature. This probably occurs because the retrospective studies frequently employ FFPE liver tissue and DNA extraction is a limiting factor in this type of sample. However, several researchers have detected *H. pylori* and its virulence factors (*vacA* genes and 26 kDa) in paraffin embedded liver samples [21, 27, 34, 47, 48].

Laser capture microdissection (LCM) is a technique that has recently become available for isolation of individual or groups of cells from a heterogeneous tissue sections by microscopic visualization. The technique was first described in 1996 by researchers of the National Institutes of Health (NIH) in Bethesda, MD [49] and allows the isolation of cells reducing the interference from nontarget cell population. The method allows selection of unmixed starting material for DNA, RNA or protein extraction for further downstream analyses [50].

The LCM system is based on an inverted light microscope fitted with a laser device to facilitate the visualization and procurement of cells [51]. The PALM MicroBeam System (Carl Zeiss, MicroImaging GmbH, Göttingen, Germany) was used in this study and it is based on the Laser Microdissection and Pressure Catapulting technology. This system consists of an inverted microscope with a motorized stage and a pulsed “cold” nitrogen ultra-violet (UV) laser. The laser is focused through the objective lenses to a micron-sized spot diameter. The narrow laser focal spot allows the ablation of the material while the surrounding tissue remains fully intact. The microscope stage and UV laser are controlled by a PC, and a video camera allows for tissue sections to be displayed on the PC screen. Cells or regions of interest are then identified and manually delineated on the computer screen using the software program. The

microscope is then instructed to collect delineated regions. The noncontact capture of homogeneous tissue samples or individual cells is achieved by means of catapulting using PALM's patented Laser Pressure Catapulting technology. With the same laser, the separated cells, or the selected tissue area, can be directly catapulted into the eppendorf cap containing a depressed lid [52, 53]. The Figure 1 shows the LCM technology employed in this study.

LCM has been used in a wide variety of applications, including pathology [54, 55], organ transplantation [56, 57], gene expression [52, 58] and molecular characterization of cancer cells [59, 60, 61]. LCM is compatible with most stains and tissue preservation techniques including frozen sections, FFPE tissues, cytology preparations and cultured cells [52, 62]. Because of its high precision and accuracy LCM has been successfully employed to isolation of bacterial cells in FFPE tissues including *H. pylori* [48, 61, 62, 64, 65].

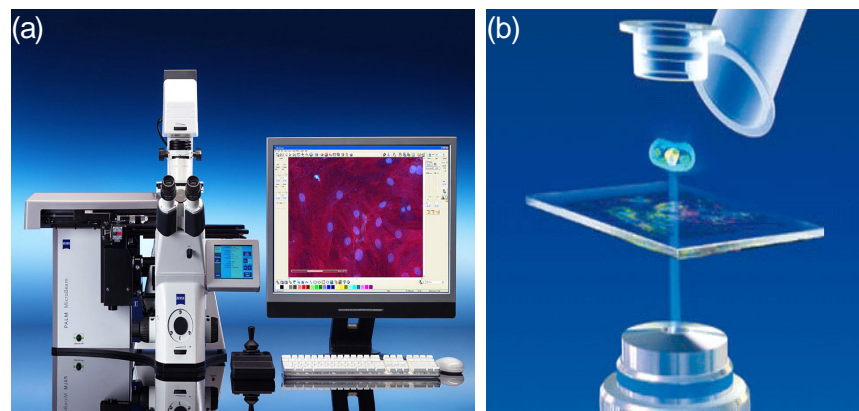


Figure 1. Images of LCM technology used in the study. In A is represented the complete PALM MicroBeam system. In B is illustrated the path of the laser beam passing through the objective lens to reach the tissue slice and the catapulting process for capturing the cells of interest into the Eppendorf cap. Source of the images: www.zeiss.de.

2. Clinical samples, methods and results

2.1. Clinical samples

This study was carried out utilizing six cases of FFPE liver tissue from patients with HCC from Department of Anatomic Pathology, Faculty of Medical Sciences, State University of Campinas (UNICAMP), Campinas, São Paulo, Brazil. The mean ages of patients was 56.0 years, with 4 male cases (66,6%) and 2 female cases (33,4%). All the fragments of liver were obtained during hepatic surgery (either transplantation or partial hepatectomy). These samples were *H. pylori* positive previously detected by polymerase chain reaction (PCR) with *H. pylori* specific 16S rRNA primers [27]. The selection criteria for the paraffin blocks included specimens archived for 5 years (2008 to 2012). The present study was approved by the Ethics Committee of the Faculty of Medical Sciences, UNICAMP (CEP 616/2009).

2.2. Methods

2.2.1. Tissue preparation for LCM

Six *H. pylori* positive samples were cut into 10µm-thick sections and mounted on 0.17mm PEN membrane-covered slides (Carl Zeiss, MicroImaging GmbH, Göttingen, Germany). After slicing, the sections were placed at 60°C for 30 minutes, then deparaffinized in 3 xylene baths (3x1min), rehydrated in decreasing alcohols (100%, 95% and 70%, each for 30 seconds) and washed for 30 seconds in tap water. Further, the routine staining with carbol fuchsin was performed [66]. At this step the sections have remained in the dye for at most 15 seconds. The stained sections were observed under the microscope for the identification of bacteria.

2.2.2. Bacterial microdissection

Stained bacteria were microdissected using a PALM MicroBeam system (Carl Zeiss, MicroImaging GmbH, Göttingen, Germany) (Figure 1A). The areas with target bacteria were traced around and microdissected together with the pieces of thin membrane by laser microbeam and then ejected into the Eppendorf tube cap by a single laser shot (Figure 1B). The tube was stored at -80°C until DNA extraction.

2.2.3. DNA extraction

After microdissection, the cap was inserted into an Eppendorf tube containing 100µl digestion buffer, prepared with 10mM Tris-HCl (pH 8.0), 1mM EDTA, 1% Tween 20 and 0.3% proteinase K. After that, samples remained in a water bath at 56°C for 3 hours and the tube was heated to 95°C for 5 min to inactivate proteinase K. The crude lysate was directly employed as template for PCR. All of these procedures were previously described with minor modifications [48].

2.2.4. PCR amplification

The samples were further amplified by PCR using *H. pylori* 16S rRNA primers. The sequence of the sense primer (JW21) was 5'-GCGACCTGCTGGAACATTAC-3' (position 691-710) and the antisense primer (JW22) was 5'-CGTTAGCTCCATTACTGGAGA-3' (position 829-809) and they amplified a product of approximately 129bp [27]. Briefly, 1µl of DNA extracted was added to 25µl PCR mix containing deoxynucleoside triphosphates (dNTPs) at concentrations of 200 µM each, 2.0µl of 25mM MgCl₂, 0.25 µl of GoTaq Hot Start Polymerase (Promega Corp., Madison, WI, USA), 4.0 µl of 5X GoTaq Flexi Buffer (supplied with the enzyme) and 20 pmol each primer (Life Technologies, Carlsbad, CA, USA). Amplification reactions included an initial 2-minute denaturation step at 94°C, followed by 40 cycles of 30 seconds at 94°C, 30 seconds at 55°C and 45 seconds at 72°C. A final extension step for 7 minutes at 72°C was performed. The DNA extracted from *H. pylori* from FFPE gastric tissue was used as positive control and distilled water in place of the DNA samples was used as negative control for PCR assays.

2.2.5. Detection of PCR products

For analysis of the amplified products, 5 µl of the amplicons were put on 1,5% agarose gels containing 1 µg of ethidium bromide per ml. The amplicons were visualized by UV transillumination.

2.2.6. Sequence analysis

The 16S rRNA amplicons were further identified by sequence analysis using ABI Prism Dye Terminator sequencing kit with AmpliTaq DNA polymerase and the ABI 3500xL Sequencer (Applied Biosystems, Foster City, CA, USA). Sequence comparison was then carried out using the Blast program and GenBank databases.

2.3. Results

Analyzing the tissue sections stained with carbol fuchsin, we visualized microorganisms resembling *H. pylori* mainly in hepatic sinus from HCC samples. The number of cocci was greater than of bacilli (Figure 3).

Our PCR results showed that all six microdissected samples were positive for 16S rRNA gene (Figure 2) and showed 98% similarity to *H. pylori* 16S rRNA gene by sequence analysis (GeneBank accession number CP003419.1) (Figure 4).

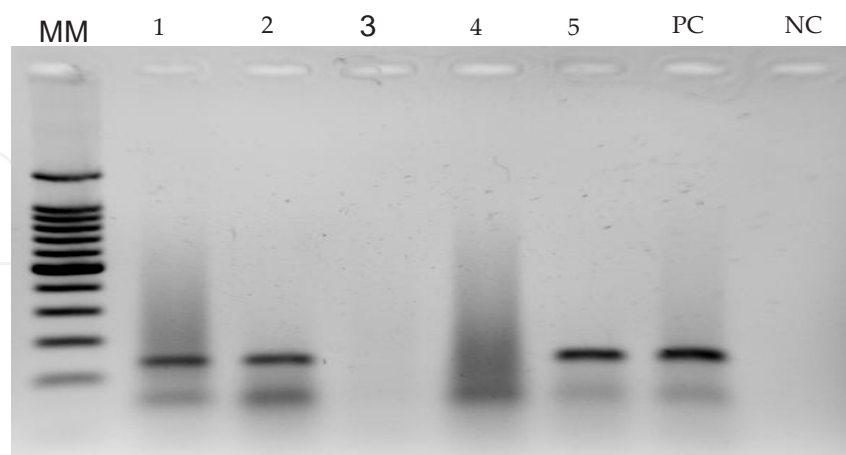


Figure 2. Results of amplification of *H. pylori* 16S rRNA gene. 1, 2 and 5 are positive samples; 3 and 4 are negative samples. MM: molecular marker, PC: positive control and NC: negative control.

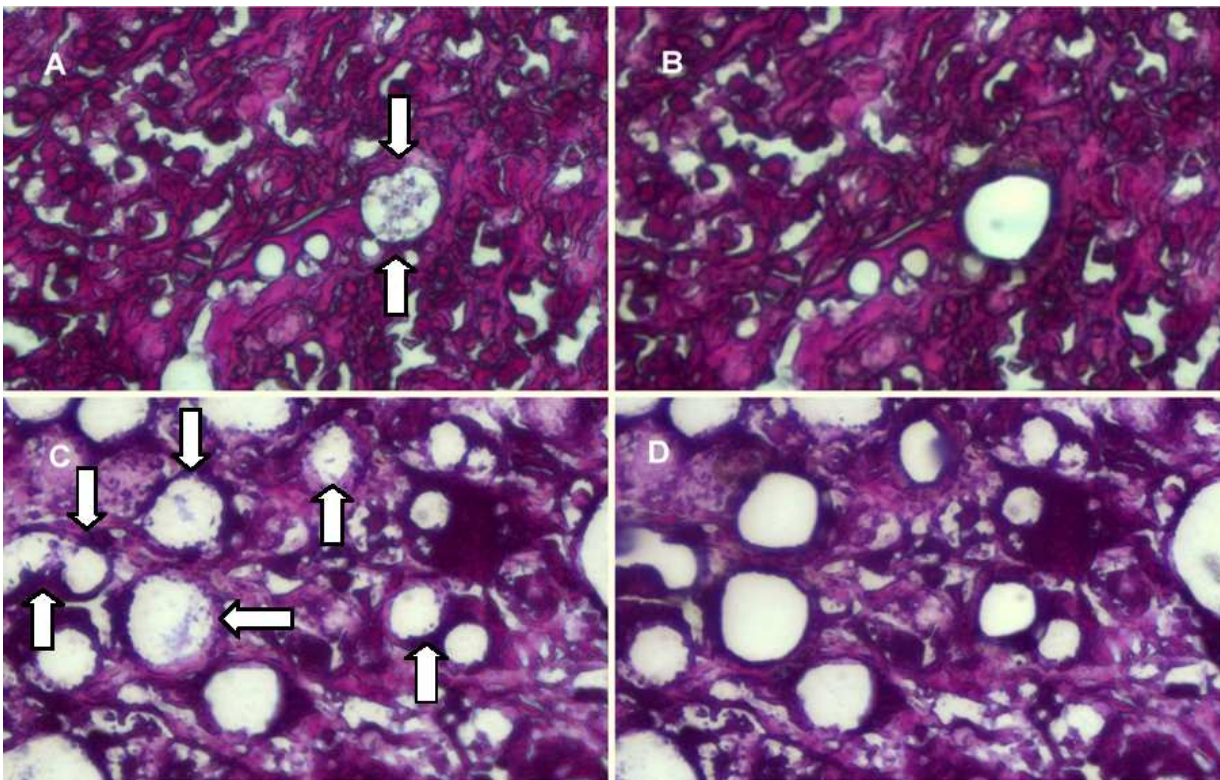


Figure 3. Optical microscopy of FFPE liver fragments of HCC patients with PCR-positive *H. pylori* 16S rRNA and stained with carbol fuchsin. In (A) and (C) bacteria are represented within sinusoid (arrows) before microdissection (magnification: 610X). In (B) and (D) the same samples are represented after bacterial microdissection (magnification: 610X).

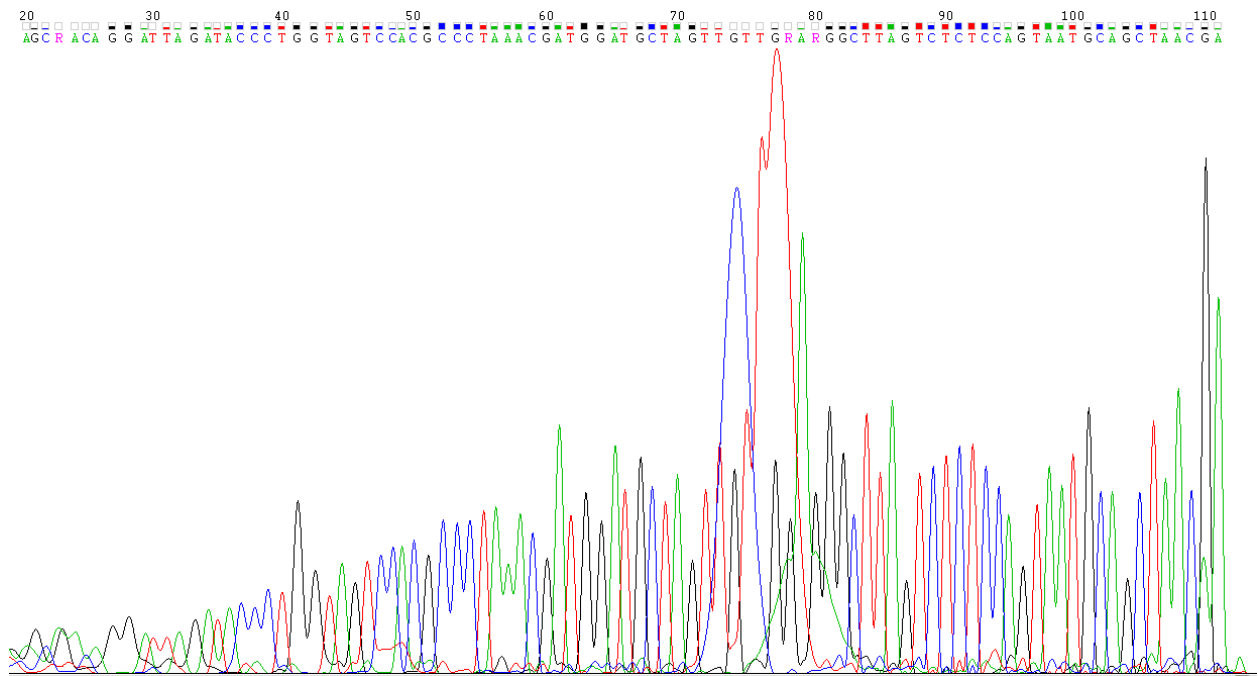


Figure 4. Electropherogram sequence of *H. pylori* 16S rRNA gene and alignment results of the BLAST databases.

3. Conclusion

The results described here confirm the identification of *H. pylori* in FFPE liver tissue from patients with HCC. Although specific primers were used for amplification of the *H. pylori* 16S rRNA gene, we cannot exclude the possibility of cross-reaction of these primers with other *Helicobacter* spp [65].

Considering the difficulty of DNA extraction from paraffin embedded samples, the use of LCM simplified the achievement of specific DNA because the DNA extraction process was reduced to a single digestion step of bacterial cells without further purification. Consequently, the crude lysate was used directly as template for PCR amplification. This is particularly advantageous when compared to traditional methods of DNA extraction that are generally laborious, toxic and time consuming [67].

Another advantage of using LCM is that it allowed the exact location of *H. pylori* in the liver, since bacteria were mainly found in the peritumoral tissue. Considering that our samples presented tumoral and peritumoral tissue in the same paraffin block, the technique was highly effective for obtaining a target bacterial population within a selected area in the HCC tissue. This is very important when we consider that the necrotic state and nuclease content of tissues may influence in recovering intact DNA specially when performing traditional methods for DNA extraction [68].

Furthermore, LCM was useful to reduce the interference from nontarget cell population considering that bacteria were found in small quantities in the liver tissue (Figure 3). In relation to nontarget cell population, it is important to consider that the major obstacle in the analysis of tumoral tissue is that it is composed by different cell types including stroma and inflammatory cells [69, 70, 71] and there is a potential dilution effect of the larger quantities of nontarget DNA found in whole tissue sections [64]. Thus, the employment of LCM was very efficient in isolating *H. pylori* despite of the reduced bacterial quantity in the HCC tissue.

In summary, we suggest that LCM can be extensively applied for identification of *H. pylori* in FFPE liver tissue. Further studies will be performed in order to amplify virulence genes of bacteria as well as to isolate *H. pylori* from other tissues using LCM technique.

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