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Rapid paper disk test for identification of *Helicobacter pylori* in mixed cultures of gerbil gastric homogenates

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ABSTRACT

A method denominated rapid paper disk test (RPDT) was developed to identify *H. pylori* colonies in complex cultures obtained from gerbil gastric homogenates. Identification is based on a characteristic reaction pattern (RP) for *H. pylori* colonies given by the combination of the urease–oxidase activities on a paper disk. Compared to the RPs obtained from gerbil's intestinal tract isolated bacteria, *H. pylori* RP is completely distinguishable, even from those of bacteria that share one or both activities as are *Aerococcus urinae, Bacillus sphaericus, Bacillus brevis, Corynebacterium pseudogenitalium,* and *Staphylococcus simulans*, as well as from those produced by collection strains *Proteus vulgaris* and *Pseudomonas aeruginosa*. This method allows the practical quantification of *H. pylori* colonies in highly contaminated plates. RPDT has the following advantages over other methodologies that use indicators in the medium: it employs two of the three routinely used *H. pylori* biochemical identification tests, the reagents do not interfere with bacterial viability, there are no restrictions in relation to the medium used, and it is a simple, fast, and low-cost method.

1. Introduction

Helicobacter pylori is a Gram negative bacteria which colonizes the human stomach and is the main cause of active chronic gastritis and peptic ulcer; besides, it has also been catalogued as a type I carcinogen by the International Agency for Research on Cancer (Kusters et al., 2006). There are many *H. pylori* identification methods that can be used in humans; some of them (culture, histology, PCR, and urea breath test (UBT), for instance) can quantify the number of bacteria in the stomach (Mégraud and Lehours, 2007).

Mongolian gerbil (*Meriones unguiculatus*) is considered an ideal animal model to establish *H. pylori* infection, because it is possible to reproduce in it the main human pathologies associated to the bacteria such as gastritis, duodenal and gastric ulcer, atrophy, intestinal metaplasia and cancer. Colonization can be produced with diverse strains, and in general infection keeps constant and spontaneous eradication does not occur (Hirayama et al., 1996; 1999; Honda et al., 1998; Ikeno et al., 1999; Keto et al., 1999; Kodama et al., 2005; Nakagawa et al., 2005; Ogura et al., 2000; Ohkusa et al., 2003; Sawada et al., 1998; Sun et al., 2003; Watanabe et al., 1998; Yan et al., 2004). Until now, the most common methods used to evaluate stomach infections are histopatological examination and culture. In culture, three standard biochemical tests (urease, oxidase and catalase), Gram stain, sensitivity to cephalothin and nalidixic acid, or PCR technique are usually used for *H. pylori* identification (Lee and Kim, 2006; Otsuka et al., 2005; Watanabe et al., 1998; Yan et al., 2004). Quantification of the number of colonizing organisms in the stomach is mainly performed by plate count, and more recently by quantitative real-time PCR (Nakagawa et al., 2005; Osaki et al., 2006; Otsuka et al., 2005; Takeda et al., 2007); with the first method *H. pylori* colony identification is tedious and delayed and, in spite of the use of selective antibiotics, contaminations appear (Kuo et al., 2008).

The Belo Horizonte medium (BHM) (Queiroz et al., 1987) as well as others with the same principle (Lee and Kim, 2006; Mabe et al., 1999; Mine et al., 2005; Nakagawa et al., 2005; Sun et al., 2003), have been used to make the plate count of gerbil stomach samples easier. In these media, 2,3,5-triphenyltetrazolium chloride (TTC) is reduced to insoluble red formazan complexes by growing *H. pylori*, giving the colonies a golden color. Although Queiroz et al. (1987) stated that this appearance is characteristic of the then-called *Campylobacter pylori* when compared with other control bacteria, currently there is not a clear knowledge explaining the phenomenon and it is necessary to apply other tests to confirm bacterial identity (Lee and Kim, 2006; Queiroz et al., 1987; Sun et al., 2003). Furthermore, there are no studies that corroborate its selectivity and sensitivity in gerbil samples.

Addition of urease reaction reagents to culture media has been proposed for bacterial identification in diverse samples (Cellini et al., 1992; Degnan et al., 2003; Testerman et al., 2001). The limitations of this method are that it is restricted to translucent media, and the reaction disseminates in large zones making colony quantification difficult.

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In this study, a new method denominated rapid paper disk test (RPDT) was developed to identify *H. pylori* colonies in mixed cultures obtained from gerbil stomachs. The method is based on two of the three routine biochemical tests used for bacteria identification. Applied to the plate count this methodology allows, in a practical and simple way, to know the number of viable *H. pylori* in gerbil's stomachs.

2. Materials and methods

2.1. Bacterial growth conditions

Helicobacter pylori ATCC 43504, *Proteus vulgaris* ATCC 6808 (a "strong urease" producer), *Pseudomonas aeruginosa* ATCC 27853 (a "weak urease" producer), as well as the isolated gerbil's gut flora were grown in different culture media, depending on the particular experimental design, as is specified later in this section. All cultures were incubated at 37 °C in a microaerophilic atmosphere (10% CO_2) in an NUAIRE incubator (NU-3500).

2.2. Mongolian gerbils

Conventional non-monitored male Mongolian gerbils (4–8 weeks old) from UNAM's Faculty of Science Biotery were used. A maximum of 6 animals per cage were housed on sawdust bedding. Tap water and pelleted diet were provided *ad libitum*. All animal experiments were performed according to the Guide for the Care and Use of Laboratory Animals of the Mexican Council for Animal Care, and the protocols were approved by the institutional Animal Care Committee.

2.3. Rapid paper disk test (RPDT) for H. pylori (patent application in process)

2.3.1. Colony transfer and urease activity

To determine the urease activity, 0.02% bromothymol blue (Merck), 0.05% phenol red (Sigma) and 6% urea (ultra pure Gibco BRL) were dissolved in distilled water and adjusted to pH 4.5 with HCl. With a thin layer chromatography reagent sprayer the solution was applied onto filter paper disks cut for Petri dishes (Whatman No. 1) and dried for 2 min. In order to transfer the colonies and test their urease activity, a disk was carefully placed on the surface of a previously grown plate and it was immediately removed. Urease positive activity was detected as a diffuse pink halo around the colonies.

2.3.2. Oxidase activity

A fresh 0.25% tetramethyl-*p*-phenylenediamine (TMPD) (Sigma) distilled water solution was applied with the sprayer onto a new paper disk. Before it dried, the disk was placed upon the first disk in which the urease test was performed. After 10–20 s, enough for the oxidase reaction to take place, the disk was separated. The test is considered positive if the colony in the urease disk turns to a violet color. The reaction pattern (RP) for positive urease–oxidase *H. pylori* colonies is a violet center surrounded by a pink halo.

2.4. Analysis of gerbil's microbiota using the RPDT

2.4.1. Isolation of gerbil's microbiota

Two gerbils were sacrificed and samples of the forestomach, forestomach bolus, stomach, stomach bolus, 2 cm of duodenum, and 40 mg of feces collected from the colon, were taken. The samples were homogenized in 2 ml of Brucella broth without antibiotics and two-fold dilutions were made (except for the duodenum and feces samples, in which the dilutions were 1:5 and 1:10, respectively). Then, 0.1 ml of the dilutions was spread onto Mueller Hinton agar plates (DIFCO) containing 5% defibrinated sheep blood (Microlab). After seven days of incubation the isolates were preliminary selected according to their Gram stain and colony morphology.

2.4.2. Reaction pattern of gerbil isolates and identification

The selected colonies were dotted on Mueller Hinton agar plates (DIFCO) containing 5% defibrinated sheep blood (Microlab), and incubated for 12–24 h. RPDT was performed in order to determine the RP. Urease positive bacteria were classified considering their bacterial group, based on the Cowal and Steel criteria, and were identified by the BBL Crystal™ GP-ID miniaturized method. In the case of non-bacterial colonies the API-20 C AUX miniaturized method was used.

2.5. Quantification of H. pylori seeded in gerbil stomach samples using the RPDT

Five 24 h fasten gerbils, were sacrificed and their stomachs were separately homogenized with 2 ml of Brucella broth medium containing different *H. pylori* ATCC 43504 concentrations (10^6, 10^4, 10^3, 10^2 CFU and a negative control without bacteria). Serial dilutions (1:10) were made and 0.1 ml of each were spread by triplicate on different media plates: Mueller Hinton, 5% defibrinated sheep blood (MH-b); Casman, 2% β-cyclodextrin (Ca-c), or Casman, 5% defibrinated sheep blood (Ca-b); all of them were supplemented with 7.5 mg/l amphotericin B, 3500 U polymyxin B sulfate, 10 mg/l vancomycin, and 5 mg/l trimethoprim. After 5 days of incubation under the previously described microaerophilic conditions, the CFU/stomach was determined by RPDT.

3. Results

3.1. Urease activity of microbiota and bacteria isolated from gerbil samples

H. pylori colonies show a characteristic RP with RPDT: a violet center surrounded by a pink halo, due to the combination of urease–oxidase activities. To determine if the method was able to distinguish between *H. pylori* colonies from other bacterial colonies sharing these activities in gerbil gastric homogenates, isolation of digestive tract microbiota and analysis with the RPDT were performed.

A total of 107 isolates were obtained, with the following proportions: 23% from stomach, 20% from stomach bolus, 26.3% from forestomach, 3.6% from forestomach bolus, 7.2% from duodenum and 19% from feces. The microscopic analysis revealed that 38.5% of the total isolates corresponded to a yeast, lately identified as *Candida guilliermondii*. This microorganism was found in all samples but was predominant in those of stomach (48%), stomach bolus (52%) and duodenum (57%).

The remaining 61.5% corresponded to bacterial cells; a selection that left 57 isolates was made based on similarities among colonies and bacterial morphology. The isolates were divided in 4 groups depending on their growing time: 18, 24, 48 and 120 h. Analysis by RPDT showed that only 8 of them were urease positive and were identified as *Corynebacterium pseudogenitalium* (isolates 58, 61, and 63), *Aerococcus urinae, Bacillus sphaericus, Bacillus brevis* and *Staphylococcus simulans* (isolates 10 and 60) (Table 1). *H. pylori* ATCC 43504 presented a similar RP to other *H. pylori* strains such as the ATCC 49503 and three human gastric isolates (data not shown), and was clearly distinguishable from the 8 non-*H. pylori* positive-urease isolated (Fig. 1, Table 1). We also compared the *H. pylori* RP with the one produced by the two collection strains *Proteus vulgaris* and *Pseudomonas aeruginosa* which are considered high-, and low-urease producing strains, respectively. In the case of *P. vulgaris* which developed a big colony in 24 h, RP was characterized by a well delimited pink colony surrounded by a less intense pink halo. These features, in addition to the lack of oxidase activity, made the *P. vulgaris* RP completely different from that of *H. pylori* (Fig. 1i *vs* k). On the other hand, *P. aeruginosa* grew in 18 h and the