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CORRELATION BETWEEN API 50 CH AND MULTIPLEX POLYMERASE CHAIN REACTION FOR THE IDENTIFICATION OF VAGINAL LACTOBACILLI IN ISOLATES

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ABSTRACT

Identification of *Lactobacillus* sp. strains by phenotypic methods may lead to doubtful results possibly interfering in the reliability of the epidemiological and probiotics studies. Therefore this study aimed to determine the best methodology for the identification of the large diversity of lactobacilli species found in the vagina by comparing two techniques, one based on their biochemical profile and other employing molecular biology. A carbohydrate fermentation test (API 50 CH) was compared with multiplex polymerase chain reaction (PCR) for the identification of species of vaginal lactobacilli from 135 healthy women. The kappa index was used to evaluate agreement between the methods. Using the molecular technique, *L. crispatus* (32.6%), *L. jensenii* (25%) and *L. gasseri* (20.6%) were the most frequent species. However, using the biochemical technique, the most frequent species were: *L. acidophilus* (34.8%), *L. crispatus* (27.2%) and *L. fermentum* (13%). Although *L. acidophilus* was the most frequent specie found by biochemical tests, no strain of this microorganism was detected by PCR. Agreement between the methods was low for identification of all the most common species. Although rates of *L. crispatus* detected were similar using both methods (32.6% and 27.2%), agreement between them was relatively low (kappa = 0.52). Conclusions: Our results confirmed the limitation of the biochemical method and the applicability of a previously published molecular method (Multiplex PCR) for the identification of lactobacilli in the vaginal tract, focusing on further necessity of its improvement for also targeting *L. vaginalis* and *L. iners*.

Key words: carbohydrate fermentation test; multiplex PCR; identification; vaginal lactobacilli; vaginal ecosystem

INTRODUCTION

Vaginal microflora of healthy women is normally composed by a large variety of aerobic and anaerobic bacteria. However, in normal flora lactobacilli species, also known as Doderlein's bacillus, are predominant and have a significant effect on vaginal microbiota (17). These microorganisms inhibit the growth of potential pathogens by competing for

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space and nutrients (3,16). Lactobacilli species also produce several antimicrobial substances such as organic acids, hydrogen peroxide (H_20_2) and bacteriocins (7). Organic acid release by lactobacilli maintains vaginal pH ≤4.5, creating an inhospitable environment for pathogens. In addition to acid production, the combination of hydrogen peroxide (H_20_2) and bacteriocins suppresses the endogenous pathogenic flora and maintains the local equilibrium (5). There are inherent differences among the vaginal lactobacilli isolates regarding their ability of conferring local protection. Since some species are known to be predominant in healthy women, presumably those are the most capable of inhibiting pathogenic bacteria. Therefore, much importance resides on the correct taxonomic identification of lactobacilli species for the development of epidemiological studies and also to enable the use of vaginal lactobacilli as probiotics (2).

Taxonomic identification by manufactured kits such as API 50 CH (bioMerieux, Craponne France), which are based on phenotypical characteristics are still widely used. *L. acidophilus* is the most common species of vaginal lactobacilli identified by this method (1). However phenotypic methods may identify lactobacilli belonging to different species as the same microorganism, which explains the variability reported by epidemiological studies performed using this methodology for lactobacilli identification (15). Currently, the definition of *Lactobacillus* species is based on the fermentative profile (phenotype), albeit practical, is known to be an inaccurate method for identification (11).

Recently, the use of genetic analyses represented an advance in the taxonomy of lactobacilli. Studies based on the DNA homology of lactobacilli concluded that some strains previously classified according to their phenotype as *L. acidophilus*, in fact consists of six different groups that cannot be differentiated biochemically. These groups were then genetically classified into six different species: *L. acidophilus*, *L. crispatus*, *L. amylovorus*, *L.gallinarum*, *L. gasseri* and *L. jensenii* (11). Later studies carried out using molecular biology revealed that *L. crispatus*, *L. gasseri* and *L. jensenii* are the most common species in vaginal environment (13).

Although identification based on DNA homology allowed better knowledge on the taxonomic relationships among lactobacilli species, the identification of vaginal isolates by this method is extremely laborious. Therefore, faster and more reliable methods need to be developed to determine the distribution of vaginal lactobacilli species (14).

There are several methods of molecular biology tools that are used for bacterial identification, such as randomly amplified polymorphic DNA (RAPD) (16), amplified ribosomal DNA restriction analysis (ARDRA), ribotyping and subsequent comparison with the ribosomal database project (RDP) (9), and sequencing and comparison with GenBank databases (www.ncbi.nlm.nih.gov) (18) or even the amplification of regions of bacterial DNA using multiplex PCR with primers that produce fragments of DNA of different sizes for each species (14).

Multiplex PCR technique uses primers that are designed based on specific sequences of bacterial DNA that codify the regions of the 16S and 23S ribosomal RNA, which are exclusive for each species and have been used successfully in the identification of intestinal lactobacilli (14). Thus, the objective of this study was to compare the identification of lactobacilli isolated from vaginal samples of healthy women using two different methods, one based on the biochemical profile using API CH50 carbohydrate fermentation test and a second one based on the multiplex PCR technique developed by Song *et al* (2000) (14).

MATERIAL AND METHODS

The study was conducted at the Department of Obstetrics and Gynaecology and Women Hospital, School of Medical Sciences, University of Campinas (UNICAMP), Campinas, Brazil. A total of 135 women at reproductive age without gynecologic diseases or complaints of vaginal discharge were

selected and included in the study. This study was approved by the local IRB, approval letter #202/2005 and all women signed an inform consent prior to enrollment. During gynaecological examination, vaginal samples were collected for the isolation of lactobacilli and for Gram-stained vaginal smears for confirmation of the absence of vaginal infections. In the Gram-stained smears, bacterial morphotypes were quantified in accordance with Nugent's criteria (10). Only women with scores from 0 to 3 were considered normal and thus included in the study.

The samples were collected in Amies charcoal transport medium and were seeded into two plates of selective medium (MRS Agar - Oxoid, Basingstoke, UK) and incubated at 37°C for 24-48 hours at anaerobic atmosphere (Forma Anaerobic System, Thermo Electron Corporation, Waltham, MA, USA)and at 5% CO_2 (Electron Series II Water-Jacketed CO_2 incubator, Thermo Electron Corporation). The isolated colonies were preliminarily identified based on their morphological and staining characteristics (Gram-positive bacilli) and their catalase reaction (negative), and stored at -70°C in MRS broth supplemented with glycerol until being submitted to the carbohydrate fermentation test using the API 50 CH carbohydrate fermentation strips (bioMerieux, Craponne, France). The results were analyzed according to the biochemical profiles registered in the APIweb® database (bioMerieux).

For molecular identification, DNA from isolates was extracted and purified using the MasterPure gram-positive DNA purification kit and Ready-Lyse lysozyme solution (Epicentre Biotechnologies, Madison, WI, USA). Identification of the species was performed by multiplex polymerase chain reaction (PCR) in two stages as described by Song *et al*. (2000) (14). Because there are many possible *Lactobacillus* sp. that can be potentially indentified and there are similarities present in their genotypes, this method consisted in a two-step process in order to compose two groups previously to the final identification. A set of primers denominated multiplex PCR-G was used to group the lactobacilli. According to the size of the amplified fragment, another set of primers, denominated Multiplex PCR-G2A and G2B, Multiplex PCR-G3 and Multiplex PCR-G4, were used to identify the species in each one of the four groups. The target species of the Multiplex PCR were: *L. delbrueckii, L. acidophilus, L. crispatus, L. gasseri, L. jensenii, L. paracasei, L. rhamnosus, L. fermentum, L. plantarum, L. reuteri* and *L. salivarius.*

The reactions were carried out in a Mastercycler gradient thermocycler (Eppendorf AG, Hamburg, Germany) with a final volume of 30µl composed of 1.5 U Taq DNA polymerase, 3 µl of reaction buffer, 2.4 µl of $MgCl_2$ (25mM), 0.24 µl of dCTP, dATP, dGTP and dTTP 25nM (Fermentas, Ontario, Canada), 1 µl of each primer (30 ng/µl) of the respective groups and 2 µl of DNA template.

The specific primers were designed based on the DNA sequences that codify the intergenic spacer region between 16S and 23S ribosomal RNA (Table 1). The amplification reactions were modified from the original publication as follows: reactions of 30 cycles that consisted of 94°C for 1 minute for denaturation; annealing at 60°C for 1 minute for multiplex PCR-G, 56°C for the others; followed by 1 minute at 72°C for extension. At the end of 30 cycles, 5 minutes at 72°C for final extension. The amplicons were analyzed by electrophoresis in 2.5% ethidium bromidestained agarose gel.

To standardize the method, 11 standard strains acquired from the American Type Culture Collection (ATCC) were used: *L. delbrueckii* (ATCC 10705), *L. acidophilus* (ATCC 4356), *L. crispatus* (ATCC 33820), *L. gasseri* (ATCC 9857), *L. jensenii* (ATCC 25258), *L. paracasei* (ATCC 27092), *L. rhamnosus* (ATCC 53103), *L. fermentum* (ATCC 14932), *L. plantarum* (ATCC 14917), *L. reuteri* (ATCC 5 53608) and *L. salivarius* (ATCC 29602).

To statistical analysis the kappa index was used to assess the comparison between the two methods and agreements were classified as follows: excellent (1.00- 0.80), good (0.79-0.60), moderate (0.59-0.40), fair (0.39-0.20), poor (0.19-0.00) or in

total disagreement (<0.0) (12).

Table 1. Sequences of oligonucleotides used in the PCRs for identification of lactobacilli in each step and corresponding species and size[14].

First step	Name	Sequence (5'-3')	Species	Product
Group I	Ldel-7	ACAGATGGATGGAGAGCAGA	*L. delbrueckii*	450 bp
	Lac-2	CCTCTTCGCTCGCCGCTACT		
Group II	LU-1P	ATTGTAGAGCGACCGAGAAG	Go to PCR 1	300 bp
	Lac-2	CCTCTTCGCTCGCCGCTACT		
Group III	LU-5	CTAGCGGGTGCGACTTTGTT	Go to PCR 2	400 bp
	Lac-2	CCTCTTCGCTCGCCGCTACT		
Group IV	LU-3P	AAACCGAGAACACCGCGTT	Go to PCR 3	350 bp
	Lac-2	CCTCTTCGCTCGCCGCTACT		
Second step				
PCR 1	Laci-1	TGCAAAGTGGTAGCGTAAGC	*L acidophilus*	210bp
	Ljen-3	AAGAAGGCACTGAGTACGGA	*L. jensenii*	700bp
	23-10C	CCTTTCCCTCACGGTACTG		
PCR 2	Lcri-3	AGGATATGGAGAGCAGGAAT	*L. cripatus*	522bp
	Lcri-2	CAACTATCTCTTACACTGCC		
	Lgas-3	AGCGACCGAGAAGAGAGAGA	*L. gasseri*	360bp
	Lgas-2	TGCTATCGCTTCAAGTGCTT		
PCR 3	Lpar-4	GGCCAGCTATGTATTCACTGA	*L. paracasei*	312bp
	RhaII	GCGATGCGAATTTCTATTATT	*L. rhamnosus*	113bp
	LU-5	CTAGCGGGTGCGACTTTGTT		
PCR 4	Lfer-3	ACTAACTTGACTGATCTACGA	*L. fermentum*	192bp
	Lfer-4	TTCACTGCTCAAGTAATCATC		
	Lpla-3	ATTCATAGTCTAGTTGGAGGT	*L. plantarum*	248bp
	Lpla-2	CCTGAACTGAGAGAATTTGA		
	Lreu-1	CAGACAATCTTTGATTGTTTAG	*L. reuteri*	303bp
	Lreu-4	GCTTGTTGGTTTGGGCTCTTC		
	Lsal-1	AATCGCTAAACTCATAACCT	*L. salivarius*	411bp
	Lsal-2	CACTCTCTTTGGCTAATCTT		

RESULTS

Ninety-two strains of lactobacilli were isolated from the 135 vaginal samples. The results according to Multiplex PCR and API are shown in Table 2. In the identification by Multiplex PCR, *L. crispatus* (32.6%) was the most common lactobacilli species in the vagina of healthy women, followed by *L. jensenii* (25%) and *L. gasseri* (20.6%). According to biochemical profiles obtained with the API test, the most frequently detected species were *L. acidophilus* (34.8%), followed by *L. crispatus* (27.2%) and *L. fermentum* (13%). Although the most frequently identified by biochemical profile (API 50 CH (bioMerieux, Craponne France) was *L. acidophilus*, we did not detect any strain of this microorganism by Multiplex PCR. Similarly, *L. gasseri* and *L. jensenii* were frequently identified by Multiplex PCR, but no strains of these species were found by the biochemical test.

Kappa index value was low for the majority of the detected species (Table 3). Although the frequency of *L. crispatus* was similar by both methods (32.6% with PCR and 27.2% with API 50 CH), the agreement rate between them was low (kappa = 0.52). Agreement between API 50 CH and Multiplex PCR for the identification of *L. salivarius* was 100% for the only case of this species identified among all the 92 species isolated in this study.

The 11 ATCC strains of lactobacilli that were used as standard controls in Multiplex PCR were also assessed by API 50 CH test and there were four cases of misidentification using this method. According to the biochemical profile, *L. jensenii* (ATCC 252580) and *L. gasseri* (ATCC 9857) were identified as *L. acidophilus*, and *L. reuteri* (ATCC 53608) as *L. fermentum*, while identification of *L. rhamnosus* (ATCC 53103) was found to be inconclusive (data not shown).

Table 2. Lactobacillus species identified according to Multiplex PCR and the API identification software database.

	Identification based on Multiplex PCR									
API 50 CH	***L. crispatus***	***L. jensenii***	***L. gasseri***	***L.* sp**	***L. delbrueckii***	***L. fermentum***	***L. reuteri***	***L. rhamnosus***	***L. salivarius***	**Total N (%)**
L. crispatus	19	5	1	-	-	-	-	-	-	25 (27.2%)
L. jensenii	-	-	-	-	-	-	-	-	-	-
L. sp	-	-	-	-	-	-	-	-	-	-
L. gasseri	-	-	-	-	-	-	-	-	-	-
L. delbrueckii	3	-	6	-	-	-	-	-	-	9 (9.8%)
L. fermentum	-	-	-	7	1	2	2	-	-	12 (13.0%)
L. reuteri	-	-	-	-	-	-	-	-	-	-
L. rhamnosus	-	-	-	-	1	-	-	2	-	3 (3.3%)
L. salivarius	-	-	-	-	-	-	-	-	1	1 (1.1%)
L. acidophilus	6	13	12	1	-	-	-	-	-	32 (34.8%)
L. brevis	-	1	-	2	-	-	-	-	-	3 (3.3%)
L. plantarum	2	4	-	-	-	-	-	-	-	6 (6.5%)
L. paracasei	-	-	-	1	-	-	-	-	-	1 (1.1%)
Total N (%)	30 (32.6%)	23 (25%)	19 (20.6%)	11 (12%)	2 (2.2%)	2 (2.2%)	2 (2.2%)	2 (2.2%)	1 (1.1%)	

Table 3. Agreement in the diagnosis of lactobacilli by molecular and biochemical methods (n=92).

	In agreement			Kappa (95%CI)	
	Negative	Positive	% of agreement		
L. crispatus	56	19	(81.5)	0.52	(0.32 - 0.71)
L. delbrueckii	81	0	(88.0)	-0.04	(-0.08 - 0.01)
L. fermentum	80	2	(89.1)	0.26	(-0.03 - 0.55)
L. gasseri	73	0	(80.4)	NP	
L. jensenii	69	0	(78.3)	NP	
L. reuteri	91	0	(98.9)	NP	
L. salivarius	91	1	(100.0)	1.00	(1.00 - 1.00)
L. sp	81	0	(88.0)	-0.04	(-0.08 - 0.01)
L. rhamnosus	89	2	(98.9)	NP	

NP = not possible to calculate
95% CI = 95% confidence interval

DISCUSSION

Agreement between multiplex PCR and API 50 CH was poor for the majority of the species. Agreement was 100% only for *L. salivarius*, which had only one single strain detected among all the evaluated species, while for *L. crispatus* agreement was 81.5%. According to the biochemical profiles by API 50 CH test applied to the APIweb® database system, the majority of vaginal lactobacilli isolates were identified as *L. acidophilus* (32.6%), which was not detected by Multiplex PCR identification. The group of lactobacilli previously classified as *L. acidophilus* was genetically evaluated and reclassified as six different species, among them *L. crispatus*, *L. gasseri*, and *L. jensenii*, which were frequently isolated in the vagina of healthy women (13). According to PCR results, *L. crispatus* was the most frequently species detected, followed by *L. jensenii* and *L. gasseri*. These findings were in agreement to other studies in which molecular techniques were used to identify lactobacilli isolated from healthy women (8, 11). Neither *L. jensenii* nor *L. gasseri*, respectively the second and third most frequently found species in this study, were identified by API, because their biochemical profiles do not exist in the database of the APIweb® system (2). Other species (*L. fermentum*, *L. delbrueckii, L. reuteri, L. rhamnosus* and *L. salivarius*) are rarely found in the vagina of healthy women, suggesting that they have fewer competitive characteristics compared to the most frequent species.

Only two species were detected with similar rates by the two methods. One was *L. crispatus* with a frequency of 32.6% by Multiplex PCR and 27.2% by API 50 CH test. Nevertheless, agreement was moderate, since only 19 strains were identified concomitantly by both methods as *L. crispatus*. *L. salivarius* was the only strain that had an agreement that was considered excellent (kappa=1.00); however, this result referred to the only strain of this species isolated in this study.

Even the reference strains used for the standardization of Multiplex PCR were incorrectly identified by the biochemical method. *L. jensenii* and *L. gasseri* were identified biochemically as *L. acidophilus*, which was already expected, since the biochemical profiles of these two species are not sufficiently different from the profile of *L. acidophilus* and are not even listed as identifiable species in this system. However, *L. reuteri* was identified as *L. fermentum* and identification of *L. rhamnosus* by APIweb® system was inconclusive.

Other studies using molecular biology techniques have reported that, in addition to *L. crispatus*, *L. jensenii*, and *L. gasseri*, other frequently found species are *L. iners* and *L. vaginalis* (2). *L. iners* was firstly described by Falsen and co-workers (6) as being a Gram-positive, facultative anaerobic rod-shaped bacterium. However, *L. iners* differs from other lactobacilli since it cannot be cultivated in MRS medium and its morphological and staining characteristics vary widely. Moreover, this species was described as consisting of a short and relatively Gram-negative bacillus (4). Since Nugent's criteria was followed for women inclusion in this study, the possible presence of women with a prevalence of *L. iners* in their flora may have been interpreted as indicative of the absence of lactobacilli and therefore excluded from the study. In addition, MRS medium was used for the cultivation and isolation of the lactobacilli and this medium would not have permitted the growth of *L. iners* possibly present in our samples.

Among the 12% of strains unidentified by Multiplex PCR, there may be a predominant species (probably *L. vaginalis*) that was not identified because there is no specific group for this species in the set of primers used in this study, which was based on methodology developed to identify species of intestinal lactobacilli. Based on these results, we intend to sequence these strains of *Lactobacillus* sp in order to identify them and use the resulting data to design specific primers for inclusion in the Multiplex PCR identification technique for vaginal lactobacilli.

In conclusion, our results confirm that the use of biochemical methods does not appear to be appropriate for the identification and study of vaginal lactobacilli, since the failure rate with this method was high compared to molecular biology techniques. In addition, our results also confirmed the applicability of Multiplex PCR for the identification of lactobacilli in the vagina, focusing on further necessary improvement of that method, targeting to *L. vaginalis* and *L. iners*.

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