ANALYSIS AND CHARACTERIZATION OF VIBRIO SPECIES ON MARINE ORGANISMS

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INTRODUCTION

Vibriosis is the name given to a group of related systemic infections in fish that are caused by marine vibrios (5, 6). Variants of vibriosis occur worldwide and affect a great variety of fish species (5), as well as crustaceans and molluscs (3, 4). Although vibriosis has been reported in complete freshwater aquaculture systems (7), this group of diseases is primarily a marine problem.

Historically, the fish-pathogenic vibrios have been assigned to the species *Vibrio anguillarum* (1, 2). This classification, however, has turned out to be an oversimplification. Efforts have been made to bring order to the heterogeneous group of fish-pathogenic vibrios by establishing serological (10, 11) and physiological (8, 9) classification systems and by describing new *Vibrio* species (such as *Vibrio salmonicida*, *Vibrio damsela* and *Vibrio ordalii*) on the basis of both phenotypic and genotypic characteristics. Despite these efforts, a satisfactory systematic classification of the fish-pathogenic vibrios has not been accomplished previously. The unresolved taxonomic affiliation of the fish-pathogenic vibrios is a problem for epidemiological studies and vaccination programs.

The goal of this study was to determine whether marine vibrios could be classified, by applying colony hybridization and restriction analysis techniques that target 16S rDNA.

GENERAL OBJECTIVE

Utilizing specific amplification, DNA-DNA hybridization techniques and restriction analysis of the 16S rDNA PCR produc s, identify species of *Vibrio* that parasitize marine organisms.

PARTICULAR OBJECTIVES AND STRATEGIES

1.- Utilizing restriction analysis, detect and characterize Vibrio species present in marine animals.

a) amplify with the universal primers, the 16S rDNA from the control and Vibrio strains.

b) make a computarized restriction analysis of known Vibrio species with the following restriction enzymes; BstU I, Msp I and Hae III. Select the best enzyme and construct a restriction library with these strains.

b) check the restriction pattern in a 2% agarose gel and compare it with the pattern of the unknown strains isolated from marine animals.

2.- Detect Vibrio species employing PCR specific amplification and DNA-DNA hybridization techniques.

a) make an alignment of the Vibrio species and select a conserve region between them to design a specific Vibrio primer.

b) make a specific PCR amplyfication of the Vibrio species with this primer and the universal forward.

c) utilizing like a probe the V. orientalis specific amplification product, detect by DNA-DNA hybridization techniques the Vibrio species.

MATERIALS AND METHODS

Bacterial strains and Growth conditions. *Vibrio* species utilized like "positive controls" *V. orientalis* ATCC 33934 T, *V. parahaemolyticus* ATCC 17802, *V. alginolyticus* ATCC 17749 T, *V. vulnificus* ATCC 27562 T and *V. fisheri* ATCC 7744 T. Bacterial strains utilized like "negative control were" *Escherichia coli* and *Bacillus subtilis*. *Vibrio* species to identify (luminiscent species isolated in the laboratory from different sea water and marine animals).

E. coli and *B. subtilis* were grown in LB medium, all the *Vibrio* species were grown and isolated at 20 C on SWC medium.

Oligonucleotides. To amplify the 16S rDNA from all the bacterial strains, I utilized the universal primers designed in the lab. To make the specific of the *Vibrio* species, the specific *Vibrio* primer was utilized (5'CCACATCAGGGCAATTTCC3').

Restriction Analysis. The 16S rRNA sequences were obtained from the GenBank and the restriction analysis was done in a 6.01 word processor. Like a results of the restriction analysis the 16S rDNA PCR products from all the analyzed strains were digested with Bstu I endonuclease and analyzed in a 2 % agarose gel.

Colony Hybridization. This technique was done utilizing the standar procedures and a fluorescent labeled probe.

RESULTS AND DISCUSSION

a) Phylogenetic analysis and 16S rDNA's amplification.

The fig. 1A shows the phylogenetic relationship between the Vibrio species analyzed and its close relatives. This tree allows to known the diversity of the samples.

The 16S rDNA PCR products of the Vibrio species selected are shown in the figure 1B.

b) Restriction analysis.

Once the 16S rRNA sequences were obtained from the GenBank database, they were imported to the 6.01 word processor and analyzed like a text to detect the restriction sites for BstU I, Msp I and Hae III enzymes (fig. 2). The number of restriction sites for each enzyme, the position and the sizes were counted and calculated manually (fig. 3A). In this figure we can clearly observe that the Vibrio species analyzed share almost the same restriction pattern , presenting small differences in size. This profile was presented only with BstU I enzyme and it is not conserve between the control 16S rRNAs (E. coli and B. subtilis). The 16S rRNA of the Vibrio species digested with Msp I and Hae III enzymes, presented a bigger and more variable number of sites, which make the analysis more difficult to interpret.

In this sense, the digestion of the 16S rDNA PCR products of the Vibrio species was done with the BstU I enzyme, and analyzed in a 2% agarose gel (fig. 3B). This figure shows that although the difference in sizes is small is still detectable. However, to improve the resolution of the restriction pattern is indispensable to utilize a polyacrilamide gel.

In addition is important to mention that in this figure the unknown luminescent strain isolated from the fishgut, presented a great similarity compared with the known Vibrio species.

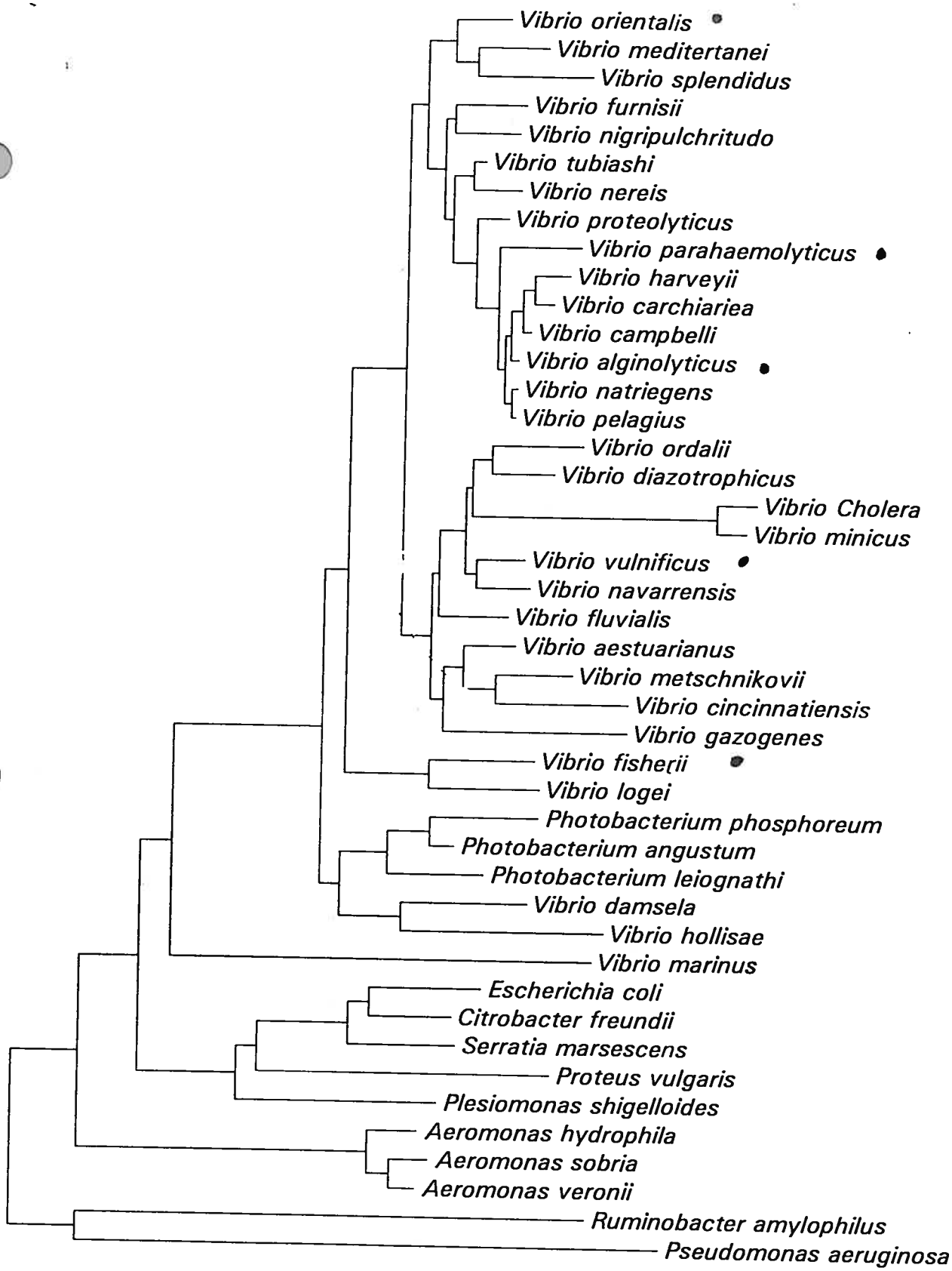


Fig. 1A.- Phylogenetic analysis of the *Vibrio Genera*.. * Vibrio species selected to this work.

Fig. 1B.- 16S rDNA PCR products from the Vibrio species (size 1,500 bp approx) . 1) V. fischeri, 2) V. alginolyticus, 3) V. vulnificus, 4) Molecular Weight Marker, 5) V. parahaemoliticus and 6) V. orientalis.

Restriction analysis of Vibrio orientalis

GAGUUUGAUCAUGGCUCAGAUUGAACGCUGGCGGCA**GGCC**UAACACAUGCA
AGUCGAGCGGAAACGACUUAACUGAACCUUCGGGGAACGUUAAGGGCGUCGA
GCGGCGGACGGGUGAGUAAUGCCUGGGAAAUUGCCCUGAUGUGGGGGAUAAC
CAUUGGAAACGAUGGCUAAUACCGCAUAAUGCCUUCG**GGCC**AAAGAGGGGG
ACCUUCG**GGCC**UCU**cgcg**UCAGGAUAUGCCCAGGUGGGAUUAGCUAGUUGGU
GAGGUAAGGGCUCACCAAGGCGACGAUCCCUAGCUGGUCUGAGAGGAUGAUC
AGCCACACUGGAACUGAGACACGGUCCAGACUCCUACGGGAGGCAGCAGUGG
GGAAUAUUGCACAAUGGGCGCAAGCCUGAUGCAGCCAUGC**cgcg**UGUAUGAA
GAA**GGCC**UUCGGGUUGUAAAGUACUUUCAGCAGUGAGGAAGGUGGAGUCGUU
AAUAGC**GGCC**UCACUUGACGUUAGCUGCAGAAGAAGCA***CCGG***CUAACUCCG
UGCCAGCAGC**cgcg**GUAAUACGGAGGGUGCGAGCGUUAAUCGGAAUUACUGG
GCGUAAAGCGCAUGCAGGUGGUUCNUUAAGUCAGAUGUGAAAGC***CCGG***GGCU
CAACCUCGGAAUUGCAUUUGAAACUGGUGGACUAGAGUACUGUAGAGGGGGG
UAGAAUUUCAGGUGUAGCGGUGAAAUGCGUAGAGAUCUGAAGGAAUA***CCGG***
UGGCGAAGGC**GGCC**CCCUGGACAGAUACUGACACUCAGAUGCGAAAGCGUG
GGGAGCAAACAGGAUUAGAUACCCUGGUAGUCCACGCCGUAAACGAUGUCUA
CUUGGAGGUUGU**GGCC**UUGAGCCGUGGCUUUCGGAGCUAA**cgcg**UUAAGUAG
ACCGCCUGGGGAGUACGGUCGCAAGAUUAAAACUCAAAUGAAUUGACGGGGG
NCCGCACAAGCGGUGGAGCAUGUGGUUUAAUUCGAUGCAA**cgcg**AAGAACCU
UACCUACUCUUGACAUCCAGAGAAG***CCGG***AAGAGAUUCUGGUGUGCCUUCGG
GAACUCUGAGACAGGUGCUGCAUGGCUGUCGUCAGCUCGUGUUGUGAAAUGU
UGGGUUAAGUCCCGCAACGAGCGCAACCCUUAUCCUUGUUUGCCAGCGAGUA
AUGUCGGGAACUCCAGGGAGACUG***CCGG***UGAUAAA***CCGG***AGGAAGGUGGGG
ACGACGUCAAGUCAUCAU**GGCC**CUUACGAGUAGGGCUACACACGUGCUACA
AUGGCGCAUACAGAGGGCAGCCAACUUGCGAAAGUGAGCGAAUCCCAAAAA
GUGCGUCGUAGU***CCGG***AUUGGAGUCUGCAACUCGACUCCAUGAAGUCGGAAU
CGCUAGUAAUCGUGGAUCAGAAUGCCACGGUGAAUACGUUC***CCGGGCC***UUG
UACACACCGCCCGUCACACCAUGGGAGUGGGCUGCAAAAGAAGUAGGUAGUU
UAACCUUCGGGAGAACGCU

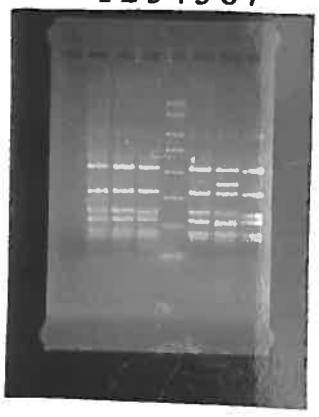
BstU I	**cgcg**	**5**
Msp I	***CCGG***	**8**
Hae III	**GGCC**	**9**

Fig. 2 .- Determination of the number and the position of the restriction sites in the 16S rRNA of the Vibrio species with the enzymes tested.

A)	CGCG		CCGG		GGCC	
	BstU I		Msp I		Hae III	
Vibrio species	# cuts	# fragments	# cuts	# fragments	# cuts	# fragments
fischeri	5	510-175 338-130 220-100	8	9	9	10
alginolyti-cus	5	"	7	8	8	9
vulnificus	5	500-184 325-125 210-108	7	8	8	9
parahaemo-lyticus	5	"	7	8	9	10
orientalis	5	"	6	7	6	7
E. coli	5	405-270 320-125 298-97	8	9	9	10
B. subtilis	7	8	9	10	4	5

B)

1 2 3 4 5 6 7



1.-V. fischeri
2.-V. alginolyticus
3.-V. vulnificus
5.-V. parahaemoliticus
6.-V. orientalis

Note: extraband in number 6 is a contamination.

Fig. 3 .-Restriction pattern of the 16S rRNA of the Vibrio species. A) Computarized restriction analysis of the Vibrio species and two control strains, E. coli and B. subtilis. B) Electrophoretic analysis in a 2% agarose gel using BstU I restriction enzyme. Lane 4; Molecular Marker. Vibrio species lanes; 1,2,3,5 and 6. Lane 7; luminescent strain isolated from the fishgut.

c) Specific amplification

To carry out the specific detection and amplification of the Vibrio species, the specific Vibrio primer was design between the region 110-150 of the 16S rRNA of these species. The sequence in this region is highly conserved between the Vibrio species with exception of V. fischeri, but is variable among species of other genera (fig. 4)

To verify the specificity of the primer, a PCR amplification at two different annealing temperatures was done. In these reaction the Vibrio specific and the universal forward primer were utilized (fig. 5A and 5B). The figure 5A shows that the specific Vibrio primer at 55 C annealing temperature, it is not too specific. This is because, the 16S rRNA controls were amplified. However, at 57 C annealing temperature this primer is highly specific and amplify only the Vibrio species (fig. 5B).

d) DNA-DNA hybridization

One of the specific PCR product obtained by the amplification with the Vibrio specific and the universal forward primer, was labeled with fluorecein with a random primer kit. This labeled product was utilized as a probe and hybridized versus a great amount of 16S rDNA products and washed at two different temperatures 40 C and 65 C (fig. 6). In this sense, like was predicted before, this probe hybridized with all the samples that contained 16S rDNA. This could be explained because all the species, share a great conserved region. However, a specific DNA-DNA hybridization could be possible utilizing like a probe the Vibrio specific primer labeled with fluorecein or radioactivity.

CONCLUSIONS

Lately, some much efforts have been done to try to implement a methodology that could be utilized to characterize a big number of samples in short time and an inexpensive way, however this efforts have not been successfully. In this sense, in this project I implemented a methodology, that can specifically, detect a group of Vibrio species and also can distinguish between them in an easy, inexpensive and fast way. This was posible utilizing a specific Vibrio primer and a restriction analysis.

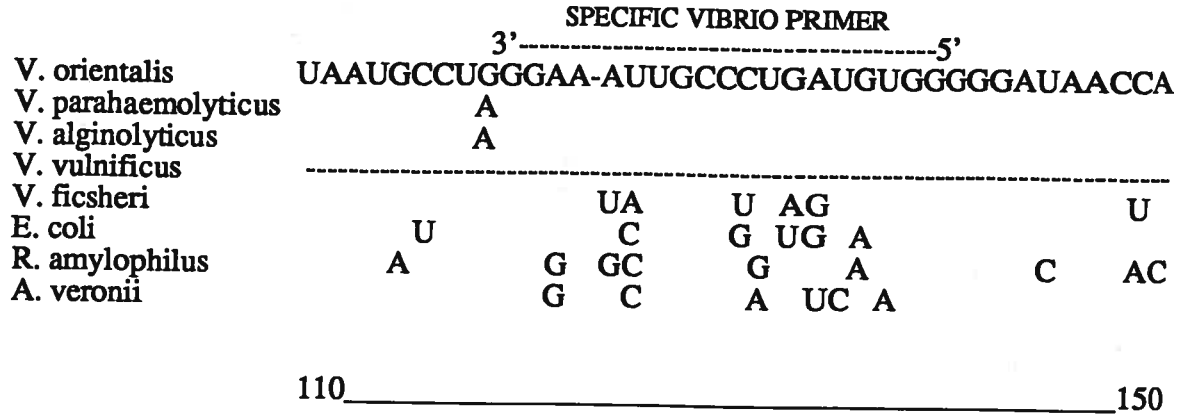


Fig. 4.- Position 110 to 150 from the 16S rRNA. This region was selected to design the specific Vibrio primer because, it is conserve between the Vibrio species analyzed, but is variable among other species.

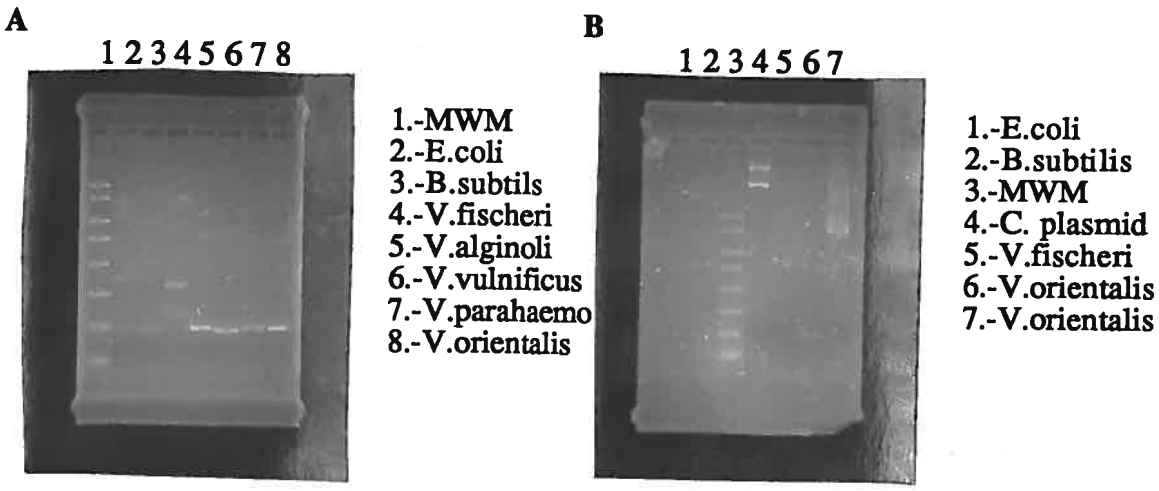


Fig. 5.-Specific amplification of the Vibrio species utilizing the universal forward and the Vibrio specific primers, 2% agarose gel. A) PCR amplification was done using 55 C as annealing temperature. B) PCR amplification was done using 57 C as annealing temperature. Lane 7; control PCR of V. orientalis utilizing universal primers. The other temperatures were 95 C and 72 C for both experiments.

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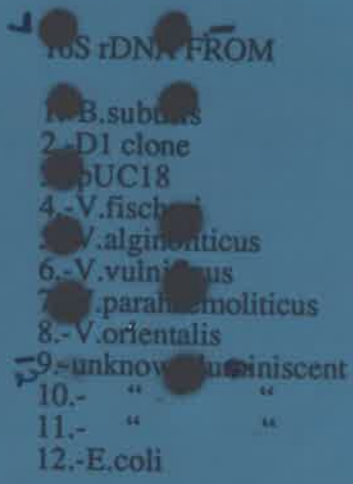
A)



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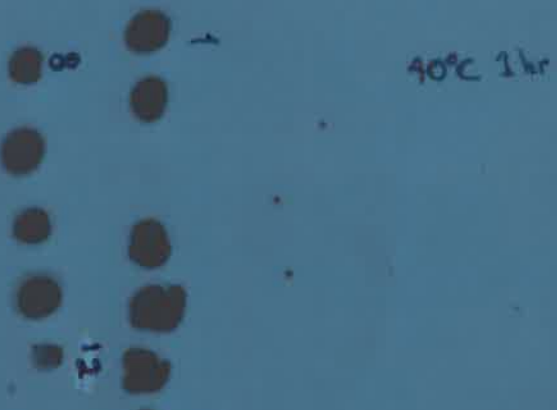

Fig. 6.-DNA-DNA hybridization at 42 C utilizing the complete 16S rDNA PCR products and the specific amplification from V. orientalis like a probe. A) Washed temperature 60 C 1hr. B) washed temperature 40 C 1 hr.

Fig. 6.-

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