DETECTION AND ENUMERATION OF *PSEUDOMONAS* IN REFRIGERATED BEEF BY COLONY HYBRIDIZATION

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Background

Several strict aerobic bacteria such as *Pseudomonas, Acinetobacter and Moraxella* are common spoilage causative organisms in aerobically refrigerated meat. *Pseudomonas*, however, is the main concern in this type of products (1). Conventional methods of characterization and counting of spoilage organisms in fresh meat are time consuming, expensive and tedious. Rapid methods are a new and exciting area in food technology particularly linked to determine on-line products and processing conditions. Hybridization techniques could be a power tool since offer high sensitivity, specificity, speed and no special equipment. These properties made this technology easy to apply in the detection of hard to grow organisms, differentiation between strains, identification of resistance genes, epidemiological studies and so on in any laboratory. All the organisms, independently of its complexity has fragments of DNA with an unique sequence, once these sequences are identified, the identification of that particular organism in a mix of many different species is easy. DNA is composed by two complementary chains of nucleotides, these strains have the property that they can be separated by heat and when the mix is cooled, the strains are bound exactly as they were before. If at the time when the DNA strains are separated and fixed over an inert support, a labeled probe with the specific sequence is added, a hybrid between the probe and the microorganism DNA is formed and this hybrid could be identified.

Objective

To develop a rapid method for the detection and enumeration of spoilage causative microorganisms in aerobically refrigerated meat.

Materials and Methods

Strains used: Different bacteria were used in order to establish the specificity of the method, they were *Acinetobacter, Aeromona, Bacillus anthracis, Brucella abortus, Brochothrix thermosphacta, Escherichia coli, Mycobacterium bovis, Moraxella, Pseudomona aeruginosa, P. putida and P. fluorescens*; all the strains were grown in BHI medium. When solid media was needed, 1.5% of agar was added.

DNA extraction: A general method (2) was used in most of the cases. In brief, 1 ml. of one over night culture were centrifuged at 12,000 rpm in a microcentrifuge, washed in 50 mM Tris-HCl pH 8.0 buffer and resuspended in the same buffer plus 50 mM EDTA and 2mg/ml of lysozyme, incubated 10 min. at room temperature; 20% of SDS-1% Proteinase K was added and incubated at 37C 30 min. DNA concentration was quantified with a DNA fluorometer.

Primers selection, probe synthesis and probe labeling: Since the sequence of the 23S fragment of the rDNA (ribosomal DNA) of *P. aeruginosa* it is known and previous work (3) shown that this fragment it is specific of *P. aeruginosa* but hybridize also with *P. fluorescens* and *P. putida*, we decided to construct a couple of primers in order to amplify this fragment to be used as probe. Using the software OLIGO (3), the following primers were selected and synthesized in a Applied Biosystems mod. 381A equipment; primer A: 5'CATGAGTAACGACAATGGGTGT; primer B: 5'GGAAGCATGGCATCAACCACTT. With these primers a PCR with chromosomal DNA of *P. aeruginosa* was done using the following conditions: 35 cycles of 1 min. at 94C for denaturation, 1 min. at 55C for annealing and 1 min. at 72C for extention. A fragment of 308bp was amplified and used as probe. This probe was labeled by random priming with ^{32}P.

Specificity of the probe: in order to check the specificity of the probe, DNA from different bacterias were dotted over a nitrocellulose membrane, denatured with 3M NaOH in a water bath at 68C, neutralized with 2M NH4OAc pH 7.0 and the denatured DNA were fixed by bake the membrane for 1 hr. at 80C.

Membrane preparation and colonies treatment: All the bacteria were grown in agar plates over night, a Nylon (S&S NYTRAN) or nitrocellulose membrane (S&S NC) was placed over the plates and when the membranes were totally wet, they were carefully removed. Two methods were used to treat the colonies over the membranes, in the first one, the membranes were placed over a 3MM Watman paper soaked with 10% SDS during 3 min., transferred to other paper with 0.5M NaOH 1.5M NaCl 5 min., transferred to other paper with 1.5M NaCl, Tris-HCl 0.5M pH 7.4 5 min. and transferred to a new paper with SSC 2X (SSC 20X= 3M NaCl, 0.3M NaCitrate pH 7.0) for 5 min. DNA was fixed to the membrane by baking at 80C 1 hr. In the second protocol, membranes were placed over a few drops of different solutions; first over 50mM Glucose, 10 mg/ml lysozyme, 50mM Tris-HCl pH 7.5, during 1 hr.; second over 0.5M NaOH-1.5M NaCl 7 min. later over 1.5M NaCl, 0.5M Tris-HCl pH 7.4, 3 min. Membranes were washed with SSC 5X and the DNA denaturalized during 20 min. over 0.4M NaOH. Cellular debris were removed by washing the membranes with SSC 5X.

Hybridization: Dot blots or colony blots were handle in the same way. The first step was a prehybridization to avoid unespecific binding of the probe, membranes were placed in a plastic bag with hybridization solution consisting of 0.1% PAES, 2.5X SSPE (SSPE 20X= 3.6M NaCl, 200mM NaH2PO4, 20mM EDTA, pH 7.4), 1% SDS and 0.01% PPiNa, during 1 hr at 65C. The probe was denaturalized by boiling in a water bath 5 min. and cooled in ice bath immediately. Probe was added to the membranes in the plastic bag at a final concentration of 5x105 cpm/ml and incubated over night at 65C or 42C. At the end of this incubation, membrane was washed twice in SSC 1X, 0.1% SDS for 15 min., twice in SSC 0.1X, 0.1% SDS 15 min. each and a last wash with SSC 0.1X without SDS. All wash were done at 65C. Membranes were dried and exposed to X ray films at -70C for 2 hs.

Naturally and contaminated beef muscle: Beef chuck naturally and artificially contaminated with *P. fluorescens* were biochemically identified as reported elsewhere (5). Selected colonies were also analyzed by hybridization.

Results and Discussion:

Specificity of the probe: Dot blot assays were done in order to check the specificity of the 308 bp probe from the rDNA of *Pseudomona aeruginosa*, it was important also, to check if this probe was able to hybridize with other species of *Pseudomonas* that also are in the group of spoilage microorganisms and also to see if does not hybridize with other microorganisms usually presents on meat. It was also important to know the better conditions for the hybridization reactions, since it is known that the temperature of wash it is important in order to increase the specificity. Figure 1 shown that the probe is good enough for *Pseudomonas* when washing is done at 65C but not at 42C, since at 42C the hybridization reaction it is also positive for unrelated bacteria.

Colony hybridization: Colonies of different bacteria were transferred to a Nylon membrane and following the methods described above, the probe was added and the hybridization carry on. Results are shown in Figure 2, the panel A is the diagram of the bacteria in the plate and the panel B are the results of the hybridization. As can be seen, only the colonies from *Pseudomonas* (*aeruginosa* and *fluorescens*) gave positive results, all other bacteria related with meat contamination gave negative results.

Comparison between biochemical and hybridization procedures: Identification of presumptive *Pseudomonas* were well correlated, when analyzed for both procedures.

Meat progressively undergoes spoilage from slaughter to consumption even if it is kept under refrigeration. Several interrelated factors, such as ph, aw, light, gaseous environment influence spoilage. Microorganisms, however, are main responsible for such deterioration. The developed hybridization procedure provides an efficient tool to detect a common meat spoilage organism, such as *Pseudomonas*, and may be used as a predictor of product shelf life.

References:

1- Gill, C.O. 1991. Microbial principles in meat processing. pags. 249-270. In: Microbiology of animals and animal products, De. J.B. Woolcock, Elsevier, Amsterdam.

2- Silhavy, T.J.; Bergman, M.L. and Enquist, L.W. Experiments with gene fusions. Cold Spring Harbor Laboratory. 1984

3- Festl, H.; Ludwig, W. and Schleifer, K.H. DNA hybridization probe for the Pseudomonas fluorescens group. Appl. Environm. Microbiol. 52:1190-1194, 1986

4- PCR Protocols. Academic Press, Inc. Harcourt, Brace, Jovanovich Publishers. 1988

5 - Lasta, J.A., Pensel, N.A., Masana, M.O., Rodríguez, H.R., and García, P.T. 1995. Microbial growth and biochemical changes on naturally contaminated subcutaneous beef adipose tissue aerobically stored. Meat Sc. 39:149-158.

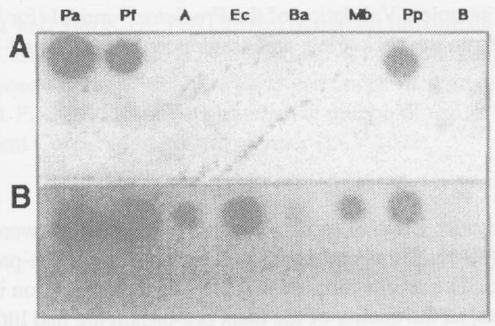

Figure 1

Dot blot hybridization of chromosomal DNA
Pa, Pseudomona aeruginosa; Pf, P.fluorescens; A, Aeromona; Ec, Escherichia coli; Ba, Bacillus anthrasis; Mb, Mycobacterium bovis, Pp, P. putida; B, Brucella abortus.
A, After hybridization, membrane was washed at 65C; B, membrane washed at 42C

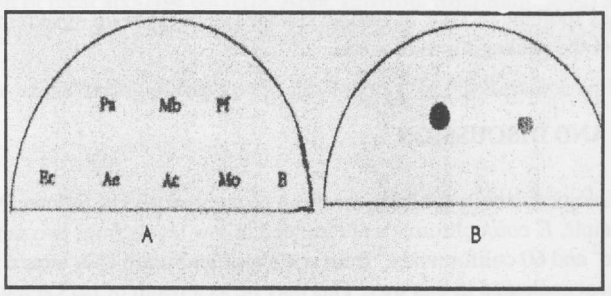

Figure 2

Colony hybridization: Pa, Pseudomona aeruginosa; Mb, Moraxella bovis; Pf, P. fluorescens; Ec, Escherichia coli; Ae, Aeromonas; Ac, Acinetobacter; Mo, Moraxela osloensis; B, Brochotrix thermoplacta.
A, pattern of the membrane; B, result of the hybridized membrane