COMPARISON BETWEEN TRADITIONAL AND PCR TECHNIQUES IN THE IDENTIFICATION OF FOODBORNE PATHOGENS IN BLOOD SAUSAGE

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Background

One of the most popular cooked blood sausage in Spain is *Morcilla de Burgos*, which is produced in the region around Burgos in the north of Spain. It consists of a mixture of onion, rice, animal fat, blood, salt and different spices such as black pepper, paprika and cumin. The product is cooked for 45-50 min at 94-95ºC, air cooled to 8-10ºC and finally chilled stored at 4ºC. This product is commercialised as a fresh product with a shelf-life of around 8-10 days, however, the use of vacuum packaging and modified atmosphere packaging has recently increased in order to extend the shelf life and widen the present market. (Santos et al, 2003). The typical way to consume this product is by deep frying in vegetable oils (mainly olive oil), roasted or boiled as part of other traditional dishes.

In a technical study made on *morcilla de Burgos* (Anonymous, 1983), it was underlie the good sanitary quality of this product, since from 67 samples analysed by traditional microbiology tests there were not detected any strain of *Staphylococcus aureus*, *Salmonella* spp., *Shigella* spp., *Escherichia coli*, or *Bacillus cereus*.

There are also another previous work that has studied the microbiology of *morcilla de Burgos*, basically the spoilage microbial population and some indicator species of the microbiological quality of the product, such as *S. aureus*, *Clostridium perfringens*, enterobacteria, pseudomonads, enterococi and moulds, without obtaining positive results in these species. (Santos 2001).

The recent advances achieved in molecular biology techniques have developed numerous genetic procedures as PCR and Real-Time PCR, which have been applied successfully to food analysis, principally in the detection of pathogenic microorganisms.

Objectives

Although there is some information about the basic microbiology of *morcilla de Burgos*, as it was mentioned above, little is known about the presence of foodborne pathogens in this product. The aim of this preliminary study is the detection of 5 species of pathogenic bacteria in this blood sausage, comparing traditional microbiology test, *versus* PCR and RT-PCR, in order to obtain a sensitive and reliable method that can assure the food safety of these kinds of products.

Materials and methods

Samples

This initial study was done with 4 *morcilla* producers, 6 samples of each one were analysed. All samples were vacuum-packed, and 3 *morcillas* were stuffed in natural beef casing and the other one in pork casing. The samples were purchased in local supermarkets and were transported to the laboratory in refrigeration conditions in iceboxes.

Microbiology assay

For detection of *Staphylococcus aureus*, *Listeria monocytogenes* and *Yersinia* spp. The pathogenic species studied were*: S. aureus* by means of homogenization of the sample and directly plated in Baird Parker agar (Oxoid LTD, Basingstoke, Hampshire, England) with supplement of egg yolk-tellurite emulsion (Oxoid), incubated at 37ºC for 72 hours; *Yersinia* spp was grown after alkaline treatment of the homogenised sample, in CIN agar (Oxoid) at 30ºC for 48 hours; *L. monocytogenes* enrichment of 20 g of sample in 180 ml of Fraser broth base (Oxoid) with ½ Fraser selective supplement (Oxoid) at 37ºC for 20-24 hours, incubation in Fraser broth base (Oxoid) with Fraser selective supplement (Oxoid) at 37ºC for 24 hours and finally cultivated in Palcam agar (Oxoid) at 37ºC for 48 hours.

PCR assays

For detection of *Salmonella* spp., *S aureus* producing enterotoxins A and D (SEA and SED), *L. monocytogenes* and *Y. enterocolítica*, 20 g of each sample was inoculated into 180 ml TSBYE medium. The samples were incubated at 37ºC ± 1ºC overnight (16 – 20 h), with shaking at 150 rpm. A portion of the sample (1 ml from the upper phase) was mixed with 1 ml of sterile phosphate buffered saline (PBS) and centrifuged at 12000 rpm for 5 min. The pellet was washed three times with PBS, then resuspended in 500 µl of water, incubated in a boiling water bath for 10 min, and immediately cooled in ice water. A 5 µl portion of each sample was added to 45 µl of PCR mixture containing 75 mM Tris HCl (pH 9), 50 mM KCl, 2mM $MgCl_2$, 0.2 mM of each dATP, dTTP, dCTP, dGTP, 0.5 µM each primer, and 1 U of Tth DNA polymerase, (Biotools DNA polimerase, Madrid. Spain.). The PCR was conducted in a Mastercycler gradient, (Eppendorf AG, Hamburg. Germany.) The amplification condition was one cycle of 94ºC for 3 min, then 30 cycles of 94ºC at 50-58ºC depending upon the primers used (see table 1) for 30 s and at 72ºC for 1 min, and finally, one cycle of 72ºC for 90 s. A positive and a negative control were used for each PCR reaction. The PCR products (10 µl of each) were separated by electrophoresis in 2% agarose gels. To stain the gel with ethidium bromide during 10 min, and the result was observed in UV light (Wang et al, 1997, Rosec at al, 2002).

RT-PCR assay

For detection of *L. monocytogenes*, samples were prepared as it was described above for PCR, 5 µl portion of each sample was added to 45 µl of PCR mixture containing 1x TaqMan universal PCR master mix (part number 4304437; Applied Biosystems, Foster City. U.S.A.), 0.3 µM concentrations of each primer, and 0.2 µM concentration of fluorogenic probe. DNA amplification was carried out in MicroAmp optical 96-well reaction plates (part number N801-0560; Applied Biosystems), sealed with MicroAmp optical caps (part number N801-0935; Applied Biosystems.). The cycling program consisted on heating for 2 min at 50°C, and then 10 min at 95°C followed by a two-stage temperature profile of 15 s at 95ºC and 1 min at 60ºC, repeated for 40 cycles. Amplification, data acquisition, and data analysis were carried out with an ABI Prism 7000 sequence detector (Applied Biosystems).

Results and discussion

Table 2 shows the results obtained for all pathogenic species evaluated. No positive samples were found for *S. aureus* enterotoxin D producer, *Salmonella* spp, and *Y. enterocolitica*, with any of the techniques used. In case of *S. aureus* producer of enterotoxin A, 10 positive samples were found by PCR, distributed between all the manufacturers, and in *L. monocytogenes* 9 positive samples were obtained by PCR and 15 positive results with RT-PCR distributed between all the manufacturers as well. Both species did not show positive results with conventional microbiology tests.

The different results obtained between traditional microbiology tests, and those of molecular biology, can be due to the different sensibility of those techniques, the use of different enrichments culture media or the growth of competitive flora. RT-PCR is 100 times more sensitive than the PCR and 1000 times more than the traditional microbiology tests used in this study. These results indicate that pathogenic bacteria detected in this study are in very low quantities. (Llanos et al, 2003).

Despite of the strong thermal treatment of 45-50 minutes at 94 º C, it is surprising, the presence of *S. aureus* producing enterotoxin A that used to be related to bad hygienic practices, and *Listeria* spp. which is an ubiquitous microorganism, and it is very extended in nature. The presence of these microorganisms can be due to a contamination in post cooking steps of cooling and manipulation during packing.

Conclusions

The molecular biology techniques used, are extremely sensitive to discover the presence of pathogenic microorganisms in low quantities *versus* the microbiological conventional technologies.

It could be very interesting to develop a more accurate HACCP system along the production line, especially in the cooling step, in manipulation before packaging, to try to keep the product in refrigeration, without breaking the cold-chain, and consuming the product according to the traditional culinary ways that imply some thermal treatment that warranties the inactivation of *S. aureus* as *L.monocytogenes*. However, other strategies, carried out in previous studies by our group, such as post-packaging short pasteurization could be very useful to obtain an acceptable sanitary quality of these kinds of products. Further studies will be developed in that sense.

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Table 1. PCR primers and working temperatures.

Species	Target gene	PCR primers´ sequences (5´-3´)	Product size	Reference	Temperature anneal (ºC)
L. monocytogenes	hemolysin gene	LM1, cggaggttccgcaaaagatg LM2, cctccagagtgatcgatgtt	234 bp	Furrer et al. (1991)	58
Y. enterocolitica	enterotoxin gene	YE1, ctgtcttcatttggagcattc YE2, gcaacatacatcgcagcaatc	159 bp	Ibrahim et al. (1992)	55
Salmonella spp.	invA gene	Sal3, tatcgccacgttcgggcaa Sal4, tcgcaccgtcaaaggaacc	275 bp	Rahn et al. (1992)	55
S. aureus	enterotoxinA gene	ESA1, acgatcaatttttacagc ESA2, tgcatgttttcagagttaatc	544 bp	Betley et al. (1998)	50
S. aureus	enterotoxinD gene	ESD1, ttactagtttggtaatatctcctt ESD2, ccaccataacaattaatgc	334 bp	Bayles et al. (1989)	50

Table 2. Results of the presence in morcilla of foodborne pathogens obtained by different analytical techniques.

Producers	Samples	*L. monocytogenes*			*Y. enterocolitica*		SEA		SED		*Salmonella* spp.
		M.A.	PCR	RT-PCR	M.A.	PCR	M.A.	PCR	M.A.	PCR	PCR
1	1	–	–	–	–	–	–	–	–	–	–
	2	–	+	+	–	–	–	–	–	–	–
	3	–	+	+	–	–	–	+	–	–	–
	4	–	+	+	–	–	–	+	–	–	–
	5	–	–	–	–	–	–	–	–	–	–
	6	–	–	+	–	–	–	–	–	–	–
2	1	–	+	+	–	–	–	–	–	–	–
	2	–	+	+	–	–	–	–	–	–	–
	3	–	+	+	–	–	–	+	–	–	–
	4	–	–	+	–	–	–	+	–	–	–
	5	–	–	–	–	–	–	–	–	–	–
	6	–	–	–	–	–	–	–	–	–	–
3	1	–	+	+	–	–	–	+	–	–	–
	2	–	–	+	–	–	–	–	–	–	–
	3	–	–	–	–	–	–	+	–	–	–
	4	–	+	+	–	–	–	+	–	–	–
	5	–	–	–	–	–	–	–	–	–	–
	6	–	–	–	–	–	–	–	–	–	–
4	1	–	+	+	–	–	–	+	–	–	–
	2	–	–	+	–	–	–	–	–	–	–
	3	–	–	+	–	–	–	+	–	–	–
	4	–	–	+	–	–	–	+	–	–	–
	5	–	–	–	–	–	–	–	–	–	–
	6	–	–	–	–	–	–	–	–	–	–

M.A.: Microbiology assay. SEA: *S. aureus* producing enterotoxin A, SED *S. aureus* producing enterotoxin D