STANDARDIZATION OF NESTED-PCR FOR THE DETECTION OF *PASTEURELLA MULTOCIDA*, *STAPHYLOCOCCUS AUREUS*, MYXOMATOSIS VIRUS, AND RABBIT HAEMORRHAGIC DISEASE VIRUS

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ABSTRACT

To control semen, oviductal fluid and embryos, as any other rabbit samples, for possible contamination of some of the most important infective elements in rabbit farms, especially in reproductive farms, four independent Nested-PCR were implemented to specifically detect reduced amounts of nucleic acids of *Pasteurella multocida*, *Staphylococcus aureus*, Myxomatosis virus, and Rabbit Haemorrhagic Disease virus in biological fluids of rabbits.

Key words: Nested-PCR, *Pasteurella multocida*, *Staphylococcus aureus*, Myxomatosis virus, Rabbit Haemorrhagic disease virus.

INTRODUCTION

Nested polymerase chain reaction (nested-PCR) is a variation of the polymerase chain reaction (PCR), in that two pairs (instead of one pair) of PCR primers are used to amplify a fragment. The first pair of PCR primers amplifies a fragment similar to a standard PCR. However, a second pair of primers called nested primers bind inside the first PCR product fragment to allow amplification of a second PCR product which is shorter than the first one. The advantage of nested-PCR is that if a wrong PCR fragment was amplified during the first amplification, the probability is quite low that the region would be amplified a second time by the second set of primers. Thus, in general, nested-PCR is a very specific PCR amplification. Furthermore, the double amplification in the nested-PCR increases the sensitivity of PCR reaction in 2-3 logarithmic units when compared with conventional PCR (Lindqvist, 1999; Marsilio *et al.*, 2005). In addition, nested-PCR has higher sensitivity than classical bacteriological or virological culture methods, but has de disadvantage that can detect non-viable infective agents. Nevertheless, a positive reaction, could help to increment the control of animals when is important to minimise the risk of diffusion of some pathogens to other farms, which is true for breeders farms or for seminal farms.

The aim of this project was to standardise nested-PCR for the detection of low contamination level of *Pasteurella multocida*, *Staphylococcus aureus*, Myxomatosis virus, and Haemorrhagic Disease virus in rabbit samples, especially semen and vitrified embryos. The infections produced by these pathogens, together with coccidiosis and Epizootic Rabbit Enteropathy, are the most important in the rabbit farms.

MATERIALS AND METHODS

Microorganisms used

To normalize the PCR conditions, and to determine the detection level of the technique, serial dilutions of 10^8 CFU bacterial suspensions obtained from 18h cultures of P-1127 strain of *Pasteurella*

multocida, and GP-484 strain of *Staphylococcus aureus*, were used. Both strains are field isolates obtained from rabbits and maintained in the bacterial culture collection of CReSA. For DNA extraction of Myxomatosis virus, Pox-Lap vaccine (Laboratorios Ovejero SA) with $10^{2.8}$ $TCID_{50\%}$ was used, and for RNA extraction of Haemorrhagic Disease virus, liver samples of an infected rabbit were used. To determine the specificity of the technique, DNA extract obtained from cultures of different strains of *Clostridium* spp., *Bacteroides* spp., *Enterococcus* spp., *Staphylococcus* spp., *Salmonella* spp., and *Escherichia coli* were used.

Nucleic acid extraction

Because PCR inhibitors are present in semen and oviductal fluid, artificially infected samples of semen and oviductal fluid were used to standardize the Nested-PCR for the four rabbit's pathogens examined. For specificity, phosphate buffer saline suspensions of heterologous bacteria were used. The DNeasy Blood & Tissue Kit (Qiagen Sciences) was used for DNA purification, and the NucleoSpin RNA virus (Macherey-Nagel) was used for RNA purification. Purified nucleic acids were immediately processed or maintained at -20°C until use.

Primers

Examining the nucleic acid data banks (NCBI) for *Pasteurella multocida*, *Staphylococcus aureus*, Myxomatosis virus (Mv), and Rabbit Haemorrhagic Disease virus (RHDv) eight series of tandem of primers were selected for the 1st and the 2nd step of the appropriate Nested-PCR. The primers used for the first step amplification of coagulase gene of *Staphylococcus aureus* (KMT1T7 and KMT1SP6) have been previously described (Townsend *et al.*, 2001), all the other primers were specifically designed for this study.

Table 1: Sequence of primers used in the Nested-PCRs for the detection of *Pasteurella multocida*, *Staphylococcus aureus*, Myxomatosis virus, and Rabbit Haemorrhagic Disease virus (RHDv), and size of the amplified fragments

Primer	Gene	Microorganism	Sequence	Amplicon Size
KMT1T7	KMT1	*Pasteurella multocida*	5'-ATCCGCTATTTACCCAGTGG-3'	460 bp
KMT1SP6			5'-GCTGTAAACGAACTCGCCAC-3'	
PmCapComAni-F			5'-TATTTTATGGCTTGTTGTGA-3'	353 bp
PmCapComAni-R			5'-CTTTTTGTTTCATTTGGACTG-3'	
CoagSau-F	Coagulase	*Staphylococcus aureus*	5'-CCGCTTCAACTTCAGCCTAC-3'	204 bp
CoagSau-R			5'-TTAGGTGCTACAGGIGCAAT-3'	
SauCoagAni-F			5'-TCAATAACCTAACGAATCCGCT-3'	96 bp
SauCoagAni-R			5'-GATGTGTATTGGTGTCGGCA-3'	
Serp2MixoMod-F	Serp2	Myxomatosis virus	5'-GTTCTCGCCGTATTATTGGA-3'	885 bp
Serp2MixoMod-R			5'-CGTGTCGTCGTGTTTGATGA-3'	
Serp2MixoAni-F			5'-TGGATAAAGTAACCGTCGCA-3'	531 bp
Serp2MixoAni-R			5'-ACTTGGGAAACGAGAGATGT-3'	
VP60RHDMod-F	VP60	RHD virus	5'-GGTTTTGCCACTGGGGCACC-3'	338 bp
VP60RHDMod-R			5'-GGTTGGGAGCCTGTGCCGTA-3'	
VP60RHDAni-F			5'-CCCACCACCAACACTTCAGG-3'	212 bp
VP60RHDAni-R			5'-GGTGTGTTCTTACCCACAGG-3'	

General nucleic acid amplification conditions

For the nucleic acid amplification a GenAmp 9700 system (Applied Biosystem) was used. To obtain the optimal annealing temperature for each tandem of primers a T-Gradient system (Biometra) was used. For bacteria, Myxomatosis virus, and the second amplification step of RHD virus, the PCR reaction was composed of 2 µl purified DNA or first step PCR-amplified product, 2 µl of each primer, 25 µl of AmpliTaq Gold PCR Master Mix (Applied Biosystem) and 19 µl of deionised water.

For the first PCR amplification of RNA of RHD virus, 10 µl of RT-PCR Buffer (Sigma), 3.5 µl of 25 mM $MgCl_2$, 4 µl of 10 mM dNTP mix, 0.5 µl of RNase inhibitor, 100 mM DTT, 0.5 µl of each primer,

0.5 µl of AmpliTaq Gold DNA Polymerase, 0.5 µl of MultiScribe Reverse Transcriptase, 1 µl of RNA sample, and 26.7 µl of deionised water. Different temperatures, and time intervals, were examined to obtain the optimal conditions for the highest specificity and sensitivity of PCR reactions.

Analysis of the amplification products

The amplified products were maintained below 4°C until use. The amplified fragments were examined by agarose gel electrophoresis. Electrophoresis at 150V during 30 minutes in 2% agarose (Sigma) in TBE buffer (Sigma) was used to determine the size of the amplified fragments. Step Ladder 50 bp (Sigma) was used as relative size marker. Gels were examined with a ChemiGenius (Syngene) system and the GeneTools (Syngene) software.

RESULTS

PCR conditions

After the analysis of different temperature and time conditions, the PCR conditions for the improved results of the four Nested-PCRs were:

- For the first and the second PCR amplification of KMT1 gene of *Pasteurella multocida* the thermal-cycler programme was: 95ºC/5'; (95ºC/30"; 58ºC/30"; 72ºC/1'45")x30; 72ºC/15'; 4ºC/∞.
- For the first PCR amplification of coagulase gene of *Staphylococcus aureus* the thermal-cycler programme was: 94ºC/4'; (94ºC/1'; 63ºC/1'30"; 72ºC/1'30")x35; 72ºC/15'; 4ºC/∞.
- For the second PCR amplification of coagulase gene of *Staphylococcus aureus* the thermal-cycler programme was: 94ºC/4'; (94ºC/1'; 60ºC/1'; 72ºC/1'15")x35; 72ºC/15'; 4ºC/∞.
- For the first PCR amplification of Serp2 gene of Myxomatosis virus the thermal-cycler programme was: 94ºC/4'; (94ºC/1'; 65ºC/1'; 72ºC/1'15")x35; 72ºC/15'; 4ºC/∞.
- For the second PCR amplification of Serp2 gene of Myxomatosis virus the thermal-cycler programme was: 94ºC/4'; (94ºC/1'; 65ºC[Δ-0.2ºC/cycle]/1'; 72ºC/1'15")x35; 72ºC/15'; 4ºC/∞.
- For the first PCR amplification of VP60 gene of RHD virus the thermal-cycler programme was: 42ºC/12'; 95ºC/10'; (94ºC/20"; 55ºC/1'; 62ºC/1')x43; 72ºC/7'; 4ºC/∞.
- For the second PCR amplification of VP60 gene of RHD virus the thermal-cycler programme was: 94ºC/4'; (94ºC/1'; 70ºC[Δ-0.2ºC/cycle]/2'; 72ºC/1'15")x35; 72ºC/15'; 4ºC/∞.

Specificity and sensitivity

Figure 1 shows the specificity of the normalized nested-PCR using the primers for *Pasteurella multocida* (A), *Staphylococcus aureus* (B), Myxomatosis virus (C) or Rabbit Haemorrhagic Disease virus (D). Only specific bands were observed in the homologous lane: 353 bp for *Pasteurella multocida* in gel A lane 20, 96 bp for *Staphylococcus aureus* in gel B lane 21, 531 bp for Myxomatosis virus in gel C lane 22, and 212 bp for RHD virus in gel D lane 23.

No unspecific amplifications were obtained when suspensions of *Clostridium paraputrificum* (lane 1), *Clostridium tetani* (lane 2), *Clostridium perfringens* (lane 3), *Bacteroides vulgatus* (lane 4), *Bacteroides* spp. (lane 5), *Bacteroides thetaiotaomicron* (lane 6), *Bacteroides fragilis* (lane 7), *Enterococcus faecium* (lane 8), *Enterococcus hirae* (lane 9), *Enterococcus faecalis* (lane 10), *Escherichia coli* (lane 11), *Staphylococcus cohnii* (lane 12), *Staphylococcus saprophyticus* (lane 13), *Staphylococcus* spp. (lane 14), *Staphylococcus sciuri* (lane 15), *Staphylococcus xylosus* (lane 16), *Staphylococcus auricularis* (lane 17), *Salmonella* Typhimurium (lane 18), and *Escherichia coli* (lane 19) were used.

The sensitivity level for the Nested-PCR of *Pasteurella multocida* was 10^1 CFU/2 µl of sample of the purified DNA. For *Staphylococcus aureus* the sensitivity degree was 10^2 CFU/2 µl of sample of the purified DNA. For Myxomatosis virus the sensitivity degree was 10^{-3} $TCID_{50\%}$/2 µl of sample of the

purified DNA. Finally, the sensitivity level for the Nested-PCR of RHD virus was 10^0 $LD_{50\%}$/2 µl of sample of the purified RNA.

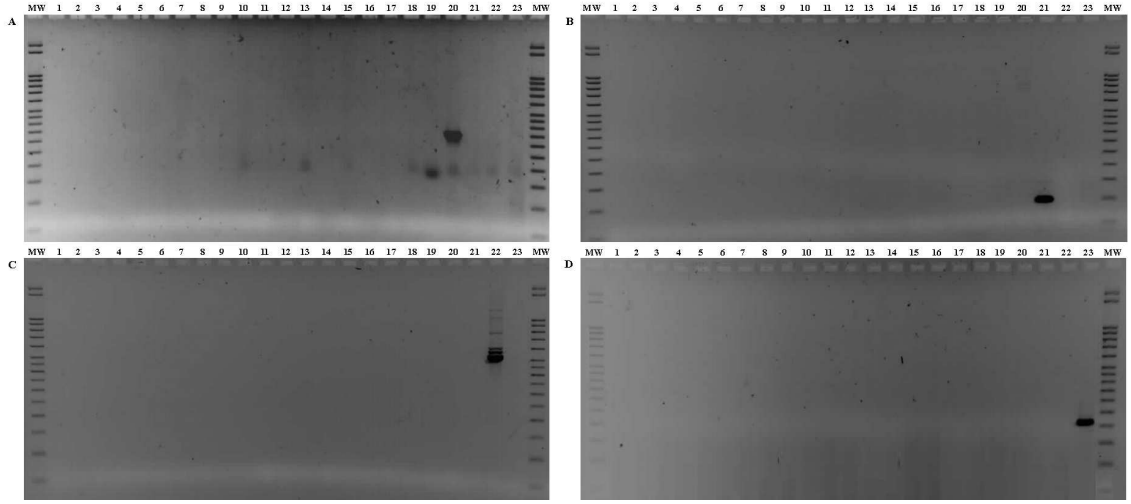

Figure 1: Electrophoretic profile of amplification products after the second PCR procedure of Nested-PCR using the primers for *Pasteurella multocida* (A), *Staphylococcus aureus* (B), Myxomatosis virus (C), and RHD virus (D)
[MW= Step Ladder 50 bp (Sigma), *Clostridium paraputrificum* (1), *Clostridium tetani* (2), *Clostridium perfringens* (3), *Bacteroides vulgatus* (4), *Bacteroides* spp. (5), *Bacteroides thetaiotaomicron* (6), *Bacteroides fragilis* (7), *Enterococcus faecium* (8), *Enterococcus hirae* (9), *Enterococcus faecalis* (10), *Escherichia coli* (11), *Staphylococcus cohnii* (12), *Staphylococcus saprophyticus* (13), *Staphylococcus* spp. (14), *Staphylococcus sciuri* (15), *Staphylococcus xylosus* (16), *Staphylococcus auricularis* (17), *Salmonella* Typhimurium (18), *Escherichia coli* (19), *Pasteurella multocida* (20), *Staphylococcus aureus* (21), Myxomatosis virus (22), and RHD virus (23)]

DISCUSSION

In this work we describe the optimal nested-PCR conditions for the detection of two of the most important bacteria and the most important virus of rabbits, with the objective of improve the detection of these pathogens in samples with low contamination levels, but epidemiologically important, i.e.: seminal doses for artificial insemination, vitrified embryos for embryos transfer. The proposed nested PCRs are highly specific, because other bacteria that can contaminate semen or embryos were not detected. Nevertheless, other not included bacteria and virus must be examined to verify the specificity.

The sensitivity on the proposed nested PCR was similar to other nested PCR for bacteria and virus: for Gram-negative bacteria, the nested PCR was able to detect at the single-cell level (Hashimoto *et al.*, 1995), and for Gram-positive bacteria the detection level is located in 10^1 CFU (Mayoral *et al.*, 2005); for virus, a nested PCR for the detection of Bovine herpesvirus-1 (BHV-1) in bovine semen resulted in a detection level of 10^{-3} $TCID_{50}$/50 µl (Rocha *et al.*, 1998).

As in other PCR methods, the system detects intact gene sequences and no viability of pathogens, because that this method can result as positive in un-infective material but reveals the previous positiveness of sample.

CONCLUSIONS

Nested PCR amplification for the most important infectious agents of rabbits could be a useful method to reduce the possibility of infection of rabbitries with *Pasteurella multocida*, *Staphylococcus aureus*,

Myxomatosis virus, and Rabbit Haemorrhagic Disease virus carried by rabbit's fluids, as semen, or by rabbit's embryos.

ACKNOWLEDGEMENTS

The study has been supported by the grant RZ2004-00015 and INIA-FEDER.

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