Adaptation of SCOTS method for *Ehrlichia ruminantium*: Innovative approach for transcriptomic analysis of obligatory intracellular pathogen

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Introduction

The Rickettsia *Ehrlichia ruminantium ER* is the causative agent of heartwater, which affects wild and domestic ruminants and is transmitted by ticks of the genus *Amblyomma*. Recently, the genomic organisation of this microorganism was revealed by the sequencing of two strains: Gardel and Welgevonden (1). Genes involved in virulence, host cell penetration or invasion and intracellular growth process are still unidentified. Transcriptomic analysis using high throuput microarray analysis and Q RTPCR will be done in order to identify the genes involved in the attenuation and the *ER* pathogenis. A large amount of RNA from virulent and attenuated Gardel strains can be obtained using an *in vitro* model (bovine endothelial cells).
Due to the obligate intracellular properties of *ER*, the main obstacle to do accurate transcriptomic study is the production of purified *ER* transcripts without host cell contaminant. Selective capture of transcribed sequences (SCOTS) was originally developed by Graham and Clark-Curtiss in 1999 for the non obligatory intracellular pathogen *Mycobacterium tuberculosis* (2), and was further used for transcriptomic microarray analysis (3). In this study, we report for the first time the successful adaptation of the SCOTS method to an obligate intracellular bacterium.

Results

NkpNI PCR after SCOTS

After each capture, a PCR using NKPNI primer was performed to confirm the efficiency of the cDNA hybridization to gDNA and then the selective captures. Figure 1 shows amplicons after NKPN1 PCR obtained for Gardel passage 39 at 96 and 120 hpi. There is a diminution of the size of the amplified transcribed sequences after each capture

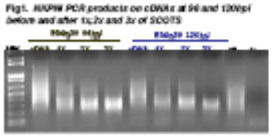


Fig1. *NKPNI PCR products on cDNAs at 96 and 120hpi before and after 1x,2x and 3x of SCOTS*

1X, 2X and 3X: Amplification signals correspond to results obtained by NKPNI-PCR on cDNA after one, two and three rounds of capture.
cDNA: Amplification signals correspond to results obtained NKPNI-PCR on total cDNA generated before SCOTS.
MM: Molecular weight 100pb DNA ladder

Enrichment of *ER* cDNA after SCOTS

Southern blots were done on cDNAs before and after each capture for each time of infection for virulent Gardel. Figure 2 shows southern blots obtained using cDNA at 96 hpi and 120 hpi as probes on *ER* gDNA . DIG-rDNA 23S+5S and 16S are used to reveal the bands corresponding to *ER* rDNA (lane 1). We observe a decrease of contaminant rRNA after the second and third capture. After successive capture, there is a progressive increase of bacterial cDNA in diversity and in amount. Similar results were obtained at different time of infection.

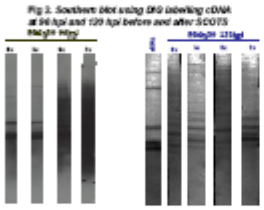
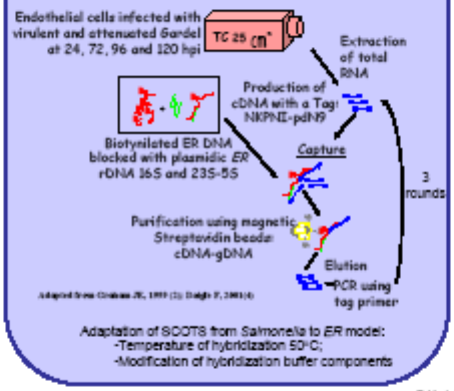


Fig 2. *Southern blot using DIG labelling cDNA at 96 hpi and 120 hpi before and after SCOTS*

1X, 2X and 3X: Southern blots showing bacterial cDNA obtained from endothelial cells after one, two and three rounds of SCOTS respectively at 96hpi and 120hpi.
dc: Southern blot showing bacterial cDNA before SCOTS in initial sample at 96hpi and 120hpi respectively.
rDNA: Southern blot showing *ER* ribosomal DNA 16S, 23S and 5S hybridization probes.

SCOTS method for production of cDNA samples from ER



Adaptation of SCOTS from Salmonella to *ER* model:
-Temperature of hybridization 50°C;
-Modification of hybridization buffer components

Detection of *ER* transcripts by RTPCR after SCOTS

Detection of transcripts of 5 *ER* genes were done following successive capture to assess the increase in ER specific transcripts (Figure 3 and Table 1)
-there was no detection of *ER* transcripts before capture for all genes
-there was an increase of the amount of cDNA for each target genes after each capture
-3 rounds of capture were necessary to obtain a maximal amount of cDNAs for variable expressed genes (table 1)

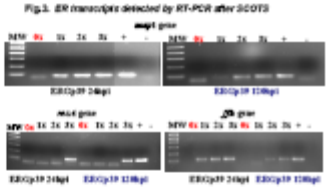


Fig 3. *ER transcripts detected by RT-PCR after SCOTS*

1X, 2X and 3X: amplification signals correspond to results obtained by RT-PCR on cDNA after one, two and three rounds of capture.
dc: amplification signals correspond to results obtained RT-PCR on total cDNA generated before SCOTS
MM: Molecular weight 100pb DNA ladder

Detection of *ER* 16S by RTPCR after SCOTS

Detection of ER 16S by RTPCR to assess the decrease in contaminant transcripts: There was a significant diminution of 16S contaminant following successive capture for 96 and 120 hpi (table 2).

Table 2. Quantitative RT-PCR of specific ribosomal 16S gene of *ER*

	Samples	16S rcDNA copy	Cycle threshold
	Before SCOTS	18233	21
96 hpi	1x	1689	24
	2x	2684	23
	3x	1265	24
	Before SCOTS	3965	23
120 hpi	1x	1586	24
	2x	92	28
	3x	43	28

Table 1. Detection of *ER* specific genes by RT-PCR on total cDNA before / after SCOTS

Gene per infection	24hpi			72hpi			96hpi			120hpi		
Gene	Before SCOTS	1X	2X	3X	Before SCOTS	1X	2X	3X	Before SCOTS	1X	2X	3X	Before SCOTS	1X	2X	3X
map1	-	+	+++	+++	-	+	++	+++	-	+	++	+++	-	+	++	+++
recA	-	-	-	+	-	+	++	+++	-	+	++	+++	-	-	-	+
rpoD	-	-	-	++	-	+	++	+++	-	+	++	+++	-	-	-	+
ffh	-	+	+++	+++	-	+	++	+++	-	+	++	+++	-	+	++	+++
pCS20	-	+	+	+	-	+	+	+	-	+	++	+++	-	+	++	+++

(-) : absence of amplicon after RTPCR,
(+) : presence of amplicon after RTPCR.
1X, 2X and 3X correspond to results obtained on cDNA after 1, 2 or 3 rounds of capture.

References

1. Frutos et al., Journal of Bacteriology, 2006, p2533-2542;
2. James E. Graham and Josephine E. Clark-Curtiss, PNAS 1999, Vol 96, p 11554-11559;
3. Faucher et al., PNAS, 2006, Vol 103, n° 8, p1906-1911;
4. Daigle et al., Molecular Microbiology, 2001, 41(5), p1211-1222;
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6. Dozois et al., 2000, PNAS , 100, p247-252.

Discussion & Perspectives

The innovative method of SCOTS which has been used previously for non obligatory intracellular parasites (4,5,6) was adapted to our *ER* model for further transcriptomic analysis. Hybridization temperature and hybridization buffer which conditioned an efficient hybridization in stringent conditions were modified. The diminution of the size of transcripts for NKPN1 PCR following successive captures of *ER* was observed as previously for Salmonella (4). We observed by Southern blot a progressive enrichment of *ER* cDNA between first and third capture. There was also a diminution of ribosomal contaminant after capture as shown by QRTPCR targeting 16S transcripts. Increased amounts of 5 different *ER* transcripts were observed by RTPCR following successive capture. Furthermore, this study highlighted the differential expression of map1, RecA, ffh, pCS20, rpoD since variable number of captures were necessary to detect specific transcribed. Map1 was strongly expressed whereas pCS20 was the lowest expressed gene independently of the time of infection.
Both RTPCR results and southern blot validated the successful adaptation of SCOTS method to *ER* and should enable us to perform transcriptomic study on attenuated and virulent Gardel strains. This should lead to the identification of new virulence factors.