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Original article

Serial analysis of gene expression (SAGE) in bovine trypanotolerance: preliminary results

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Abstract – In Africa, trypanosomosis is a tsetse-transmitted disease which represents the most important constraint to livestock production. Several indigenous West African taurine (*Bos taurus*) breeds, such as the Longhorn (N'Dama) cattle are well known to control trypanosome infections. This genetic ability named "trypanotolerance" results from various biological mechanisms under multigenic control. The methodologies used so far have not succeeded in identifying the complete pool of genes involved in trypanotolerance. New post genomic biotechnologies such as transcriptome analyses are efficient in characterising the pool of genes involved in the expression of specific biological functions. We used the serial analysis of gene expression (SAGE) technique to construct, from Peripheral Blood Mononuclear Cells of an N'Dama cow, 2 total mRNA transcript libraries, at day 0 of a *Trypanosoma congolense* experimental infection and at day 10 post-infection, corresponding to the peak of parasitaemia. Bioinformatic comparisons in the bovine genomic databases allowed the identification of 187 up- and down-regulated genes, EST and unknown functional genes. Identification of the genes involved in trypanotolerance will allow to set up specific microarray sets for further metabolic and pharmacological studies and to design field marker-assisted selection by introgression programmes.

SAGE / trypanotolerance / N'Dama / *Trypanosoma congolense* / transcriptomics

1. INTRODUCTION

In central and sub-Saharan Africa, the most important constraint to livestock production is trypanosomosis. This tsetse-transmitted disease represents an

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important risk for about 60 million cattle and it strongly affects their productivity (milk, meat, fertility, pulling...) on 7 million km^2 spread over 37 countries. Animal trypanotolerance is the genetic ability of some breeds from several mammalian species (such as cattle, small ruminants, pigs, wild buffaloes and antelopes...) to live normally and remain productive in tsetse-infested areas [12]. This phenomenon was described in Africa as early as the beginning of the XXth century [4, 6, 7, 29, 33, 35]. Trypanotolerance results from various biological mechanisms under multigenic control, which relate either to the control of trypanosome infection, as measured by parasitemia [10, 13, 22, 34], or the control of the pathogenic effects of the parasites, the most prominent of which is anaemia [1, 36–38]. Two different pools of genes are probably involved in determining the two characteristics and the various methodologies used so far have not succeeded in identifying them. Neither zootechnical studies [8, 9, 16, 15, 23], nor quantitative genetics approaches [39], nor the electrophoretic analysis of targeted proteins [32, 31], nor MHC typing [21] have brought significant progress on the trypanotolerance understanding. QTL studies developed more recently, firstly on mice [17–19] then on bovine [14] give some more interesting results but they are restricted to small parts of the cattle genome. Considering the limited number of experimental animals used, the confidence interval of the bovine QTLs is too wide to be useful in a marker assisted selection (MAS) programme, or in a positional candidate approach. The homologous comparison between the mouse and bovine genomes is limited and there is no proof that the same genes are involved in the two species. Finally, the QTL approach could give information on genes involved in the innate immunity but not on those controlling the acquired immunity, both gene types being involved in the global trypanotolerance mechanisms. Furthermore the crossbreeding plan to study the QTL segregation on bovines is very long and expensive. Several recent biotechnologies allow exhaustive functional analysis using a transcriptomic approach which is efficient to characterise the full complex of genes involved in the expression of specific biological functions. Amongst them, the serial analysis of gene expression (SAGE), which we used in the present work, will allow to compare up- and down- regulated genes involved in the control of *Trypanosoma congolense* infection in N'Dama cattle.

2. MATERIALS AND METHODS

2.1. Experimental animals and design

We used one animal of the N'Dama breed which is a Longhorn indigenous West African taurine (*Bos Taurus*) well known to be resistant to trypanosomosis infection. This animal was taken in the field from a highly tsetse infected area. A serological control allowed to verify the presence of specific *T. congolense*

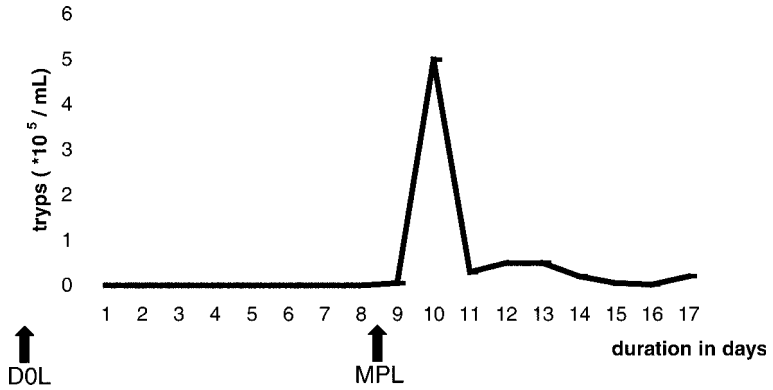

Figure 1. Evolution of parasitaemia in an N'Dama animal after a *Trypanosoma congolense* experimental infection. The arrows (↑) indicate the time when blood was sampled to constitute the D0L (Day 0) and MPL (Day 10) SAGE libraries.

antibodies. Before the beginning of the experiment, this animal was treated against blood parasites (Veriben: diminazene aceturate, 7 mg $\cdot$ kg^{-1}) and gastrointestinal parasites (Vermitan: albendazole, 7.5 mg $\cdot$ kg^{-1}). After a few days of resting, a first blood sampling was done using a PAXgene Blood RNA tube (Quiagen, cat. No 762125) which contains a total RNA conservation medium. This first blood sample at day 0 was used to develop the first reference SAGE library (D0L) from total white blood cells. Then the experimental design consisted in a *Trypanosoma congolense* infection (Ser/71/STIB/212) using a unique syringe inoculation of 8×10^5 parasites [11, 25, 27, 28]. Each couple of days, a blood parasitological control on the buffy coat allowed to check for the presence of the parasites and to follow the kinetics of their development (Fig. 1). The second blood sampling was done to develop the second reference SAGE library (MPL) at the peak of parasitaemia which appeared at day 10. These two D0L and MPL SAGE libraries were used in a differential comparison of expressed genes in this N'Dama animal before and after a *T. congolense* infection.

2.2. The SAGE method

The serial analysis of gene expression (SAGE) technique [3, 40, 41] enhances the power and the swiftness of transcriptome analysis. SAGE generates complete expression profiles of tissues or cell lines and the results are quantitative and absolute. The principle of this technique consists in the construction of total mRNA libraries for a quantitative analysis of the whole transcripts expressed or inactivated at particular steps of a cellular activation. It is based on three principles: (i) a short sequence tag (9–14 bp) obtained from a defined region within each mRNA transcript contains sufficient information to uniquely

identify one specific transcript; (ii) sequence tags can be linked together to form long DNA molecules (concatemers) that can be cloned and sequenced. Sequencing of the concatemer clones results in the quick identification of numerous individual tags; (iii) the expression level of the transcript is quantified by the number of times a particular tag is observed.

We used the I-SAGE^{TM} kit from Invitrogen (cat. No T5000–01) to develop our 2 D0L and MPL transcript libraries.

Bioinformatic comparisons [30] in several genomic databases (Unigen, Tigr) allowed firstly to identify the different activated and inactivated tags (known genes, EST or unknown genes) and secondly to compare their respective frequencies in both D0L and MPL libraries.

3. RESULTS

The analyses of the whole identified tags are summarised in Table I.

From 4763 sequenced tags, we identified 2281 distinct transcripts, 187 of them being differentially expressed in both D0L and MPL libraries. The rates of contamination by linker sequences were non-significant. Repeated ditags (not taken into account for the measurement of expression levels) represented 1.3% of the total ditag population, revealing a high complexity of the original mRNA population.

The tags showing the most significant differences in frequencies ($P < 0.001$) between both D0L and MPL libraries are separately presented for the up (Tab. II) and the down (Tab. III) regulated transcripts.

A different interesting presentation of these results is given in a graphical scatter plot (Fig. 2) where each dot represents a particular tag.

Table I. Statistics of tag distribution.

Totally sequenced tags	4763
Different tags	2281
Genes	386
cDNA/EST	920
No Match	975
Tags differentially expressed ($P < 0.001$)	187
Genes	92
cDNA/EST	23
No Match	72

"Genes" are tags matching with well identified genes; "cDNA/EST" are tags matching with anonymous described sequences; "no match" are tags failing to match with SAGEmap (rank 1 or 2) or UniGene sequences.

Table II. Expression of UP-regulated transcripts. (D0L): day 0 non infected library; (MPL): maximum parasitaemia library; (Id.): databases accession number; No Match: unknown genes. Significance of differences between D0L and MPL tags listed is at least $P < 0.05$.

Tags	D0L	MPL	Id.	Names
Immunity proteins				
CATGGACCCCTGAG	27	99	Bt.100316	Immunoglobulin light chain mRNA
CATGGAGCCCGCAG	0	24	TC132989	Ig M heavy chain constant region, membrane form
CATGAGTGCAGACT	13	18	TC132990	Ig M heavy chain constant region, secretory form
CATGGGCGTCTCTG	0	3	TC124168	Ig G3 heavy chain constant region
CATGGCCACTTAGT	0	12	Bt100505	B-cell antigen receptor mRNA
CATGTGAGGGTGCC	0	4	TC133149	T-cell receptor beta 1, constant region
CATGGATCTGGCTG	0	3	Bt.506	MHC class II BoLA-DBQ-chain mRNA
Other genes				
CATGTGACACGTAT	0	32	TC133213	NADH – ubiquinone - oxidoreductase chain 1
CATGGGCTGGGGGC	0	20	TC132831	PRO1_BOVIN Profilin I. [Bovine]
CATGCTGGGAAATT	0	13	TC132798	ORF
CATGCATATTTGGG	0	14	Bt100010	Ferritin H subunit mRNA
CATGACAACACATA	67	119	Bt.5174	Inositol polyphosphate 1-phosphatase
CATGAGGAAAGCGG	0	13	TC142407	Homologue to CDH1-D {Gallus gallus}
CATGCAGCTCCGCG	0	13	Bt.697	Cdc42-associated tyrosine kinase ACK-2 mRNA
CATGTGAGAACATT	0	12	TC132773	Actin
CATGGACCCCTTTT	0	11	TC132770	Beta actin
CATGTTGTCTGTCT	0	11	TC132625	HSHU33histone H3.3 [validated] -
CATGAGTCCAAGCC	0	11	TC143154	Similar to SDHL_HUMAN L-serine dehydratase [Human]
CATGAAGGTAATAA	0	10	TC132804	CChain C Crystal Structure Of Arp23 COMPLEX,
CATGTGTGTCTGTA	0	9	TC123714	TBA1_CRIGRTubulin alpha-1 chain. [Chinese hamster]
Ribosomal				
CATGTAAGGATCCA	0	20	TC124043	RS26_HUMAN40S ribosomal protein S26. [Rat]
CATGCTCACCAATA	13	46	Bt.101746	Ribosomal protein (QM) mRNA
CATGGGCTTCGGCT	0	14	Bt101531	Acidic ribosomal protein P2 mRNA
CATGCTGTTGGTGA	0	9	TC132795	RS23_HUMAN40S ribosomal protein S23. [Rat]
CATGAGGAAAGCGG	0	13	TC141303	Ribosomal protein L36
EST				
CATGTTGCATTACC	0	28	TC133588	EST
CATGGAGGAGGAAG	0	19	Bt.116903	EST - 211333 Bos taurus cDNA
CATGAAGCCCAGCG	0	15	Bt.95263	EST - AV662735 Bos taurus cDNA
CATGGGCTGGGGCT	0	14	TC142538	EST
CATGGCCACAGCCA	0	10	Bt77418	EST - AV603489 Bos taurus cDNA

Table III. Expression of DOWN-regulated transcripts. (D0L): day 0 non infected library; (MPL): maximum parasitaemia library; (Id.): databases accession number; No Match: unknown gene. Significance of differences between D0L and MPL tags listed is at least $P < 0.05$. *(continued on the next page)*

Tags	D0L	MPL	Id.	Names
Immunity proteins				
CATGGCTAAGCCTA	241	74	TC142442	BOVIN Beta-2-microglobulin precursor, Lactollin
CATGTTACCATAAA	13	1	Bt.100131	B.taurus mRNA for beta 2-microglobulin
CATGAGGAGTTGGG	40	20	TC133042	MHC class I heavy chain
CATGGCGCCCCTTC	27	17	Bt.100356	B.taurus mRNA for MHC class 1 (clone 6)
CATGGGCATCATTG	27	16	Bt.100358	B.taurus MHC class 1 protein molecule D18.1
CATGTCAAGGCAAT	13	1	TC144589	MHC class II DM alpha-chain MHC class II antigen
CATGTAATGCCTTT	13	8	Bt.101032	Bovine BoLA-DRA mRNA for MHC class II BoLA-DR-alpha
CATGGAAGCAATAA	13	7	TC133143	MHC class II antigen [Bos taurus]
Other genes				
CATGAATAAAGTGC	54	8	TC143206	glutathione peroxidase (AA 1–204)
CATGTACGAGAAAG	27	1	Bt.1316	B.taurus mitochondrial aspartate aminotransferase
CATGCCTCGACGAT	40	11	TC132812	cytochrome oxidase subunit[Bos taurus]
CATGCAAAGGAGAT	13	1	Bt.100650	Bovine ATP synthase inhibitor protein mRNA
CATGTAATAAAGCA	13	1	TC125185	seryl-tRNA synthetase
CATGTTAATCCTAA	13	1	Bt.5517	Bovine mRNA for retinal 2′.3′-cyclic nucle.3′-phospha
CATGCAAATAAAAA	13	1	Bt.100227	Bovine mRNA for beta-crystallin subunit beta B1
CATGCTAATTATAA	13	1	TC124062	4L NADH dehydrogenase subunit 4L [Bos taurus]
CATGCCGGCCCAGA	13	1	TC142818	Homologue to KCRB_CANFA Creatine kinase B chain. [Dog]
CATGTGTACCTTTT	13	1	TC143287	COPA_BOVIN Coatomer alpha subunit (Alpha-coat protein)
CATGGCCTGATGGG	94	51	TC133123	BAB22470.putative {Mus musculus}
CATGGGAGAAGGGT	13	1	TC142585	Similar to BAB28161. putative {Mus musculus}
Ribosomal				
CATGCACAAACAGT	40	2	TC124028	Homologue to ribosomal protein S27 cytosolic - human
CATGTGGTGTTGAG	40	8	TC123562	RS18_HUMAN40S ribosomal protein S18 (KE-3)
CATGGAACATATCC	13	1	TC132800	60S ribosomal protein L19. [Mouse] {Mus musculus}
CATGGCAGAGTTCG	13	1	TC132832	RS6_HUMAN40S ribosomal protein S6, Rattus norvegicus
CATGTGAAAGATGC	13	1	TC142426	ribosomal protein S4

Table III. Continued.

Tags	D0L	MPL	Id.	Names
EST				
CATGTAGGTTGTCT	228	107	BE236829	EST
CATGCATTCTAGAG	27	0	BF890336	EST
CATGTGAAAAAAAA	27	0	Bt.57839	EST - 170434 Bos taurus cDNA
CATGTTAATAAAAA	27	2	Bt.127590	EST - Bos taurus cDNA
CATGCGGTCAGCCA	27	3	BI540074	EST
CATGATTCTTTGGT	40	9	Bt.209523	EST - Bos taurus cDNA
CATGAACAGAGGAG	13	1	BM431775	EST
CATGGAGAAATATC	13	1	Bt.185285	EST - Bos taurus cDNA
CATGAGTTTGCCCT	13	1	Bt.6126	EST
CATGCAGCAGAAGC	13	1	Bt.95810	EST - Bos taurus cDNA
CATGAACAGAGGAG	13	1	AW463909	EST
CATGCATAAAGGAA	13	1	Bt.215670	EST - Bos taurus cDNA
CATGAACAGAGGAG	13	1	BM482666	EST
CATGCCGACGGGCG	13	1	TC123808	EST
CATGGCTGGCCTGC	13	1	TC136232	EST
Unknown				
CATGGTACATAGAC	27	0		No Match
CATGTGCTTGTCGG	13	1		No Match
CATGGTGTGATGCT	13	1		No Match
CATGTGAGAAGTCG	13	1		No Match
CATGATGAACCCTG	13	1		No Match
CATGTTTGTCATCT	13	1		No Match
CATGCAGCAAGGAA	13	1		No Match
CATGTCGGCTTCTA	13	1		No Match
CATGGTATTTGCAA	13	1		No Match

Several dots correspond to known genes (immunoglobulins, B and T cell receptors, interleukins, MHC *Bola* class I and II, metabolic and ribosomal proteins...) or EST but others correspond to unknown genes. These unknown genes could come from the N'Dama mRNA but they could also come, to a small extent, from mRNA of *T. congolense* parasites. To validate this hypothesis, we did another bioinformatic comparison of these whole no-match tags with the two available existing *Trypanosoma* genome databases (*T. brucei* and *T. cruzi*). We identified 5 expressed genes actually coming from the *Trypanosoma congolense* genome, and which are probably ubiquitous in the *Trypanosoma* genus (Tab. IV).

This result opens a very interesting way to study the interactive mechanisms at the host-parasite interface by a parallel comparison of the parasite and the host SAGE libraries.

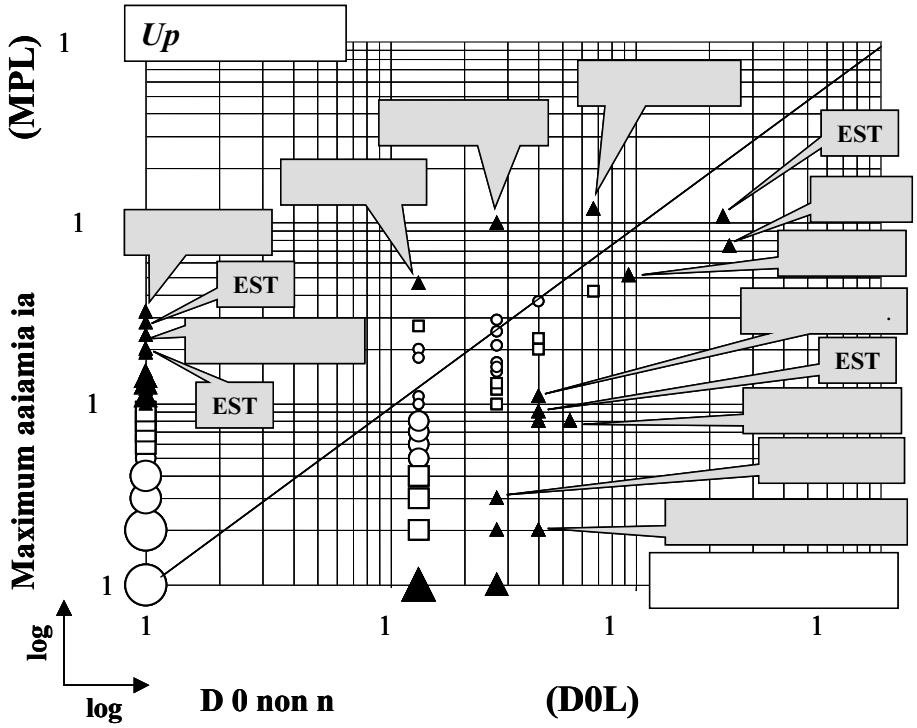

Figure 2. Comparative levels of expression in the T0 non-infected library (D0L) and in the maximum parasitaemia library (MPL). The number of occurrences of each tag was plotted on a logarithmic scale. The number of occurrences was set to one for tags with no expression in one library. Statistical significances of tag frequency differences (▲): $P < 0.001$; (□): $0.001 < P < 0.01$; (○): $P > 0.01$.

Table IV. Expression of up-regulated *Trypanosoma congolense* transcripts. (D0L): day 0 non infected library; (MPL): maximum parasitaemia library; (Id.): databases reference.

Tags	D0L	MPL	Names	Id.
CATGCCACACAAGC	0	1	TCJ3 PROTEIN. 241 3e-62	CONTIG6688
CATGTGTCACCCAC	0	1	SEPTATION. 119 8e-26	CONTIG9150
CATGGGACTTGGAC	0	1	RIBONUCLEOSIDE-DIPHOSPHATE R... 293 2e-78	EM_NEW :AL473377
CATGTGTGTCTGTG	0	1	PROTEIN PHOSPHATASE-2C. 203 2e-51	EM_NEW :AL457897
CATGTTGCTGTGTG	0	2	POSSIBLE AMINO ACID TRANSPORTER. 164 7e-77	CONTIG9490

4. DISCUSSION

Amongst the 187 regulated tags, from the pool of up-regulated transcripts (Tab. II), we found several genes involved in the immune mechanisms which confirm several previous immunological results [2]. The most activated genes were those encoding different chains of immunoglobulin (IgG and IgM) molecules. This confirms their important role in the immune mechanisms involved in the control of trypanosome infections. Indeed, the literature on this topic [2, 5, 6, 13, 20, 26] is rich of corroborating results indicating that, except for primo-infection [24], the ability of resistant animals to control parasitaemia is due to a more efficient specific antibody response. The T-independent responses producing IgM antibodies are sufficient to control the parasitaemia, and the IgM are more efficient than the IgG as neutralising antibodies at the beginning of the infection [20]. The increase of the serological IgM level and the parasitaemia appearance are simultaneous while the IgG antibodies generally appear later. The IgM are mainly directed to the parasite surface antigens while the IgG are generally directed to the internal antigens [5]. It has often been reported [22] that the trypanosomes are responsible for B and T cells polyclonal proliferation. We confirmed the activation of the genes encoding the B and T cell receptors (Tab. II). Furthermore, the T cell receptor beta cluster is located in the bovine chromosome 4 (*Bta4*) in the 4q3.1 and 4q3.6 region (*IDVGA51-TGLA159/MGTG4B*) where Hanotte *et al.* [14] described a QTL strongly associated with the fewer parasites trait (*PARMLn*) in N'Dama. We also found genes encoding cytokines such as interleukins (*IL1* and *IL10R*) confirming their role in the induction of a cell polyclonal activation, particularly for IgM antibodies [26]. Finally, MHC class II *BoLA-DQB* genes seemed to be activated (Tab. II) while other MHC genes of class I and class II (*BoLA-DRA* and *BoLA-DMA*) seemed to be down-regulated (Tab. III). Apart from molecules of the immune system, we identified several genes involved in different up- and down- regulated metabolic pathways (such as the NADH-ubiquinone oxidoreductase chain 1, the bovine profilin or the glutathione peroxidase). Several ribosomal genes were also regulated. Within regulated EST, one up-regulated (TC133588) and 3 down-regulated (BE236829, BF890336, Bt. 57839) EST are of interest, but further developments of the bovine map will be needed to clearly identify these EST. Concerning the up- and down- regulated unknown tags, they can be spotted on microarrays for further applications.

These preliminary results obtained on a single experimental N'Dama animal need to be reproduced at least on another individual of this breed. To identify the genes implicated in the trypanotolerance mechanisms, we need to implement similar differential analysis at least on two individuals from several other cattle breeds: the trypanotolerant breed (*Bos taurus*), such as Baoule, and the trypanosusceptible zebu breed (*Bos indicus*). The comparison of the results

obtained on the different trypanotolerant and trypanosusceptible cattle will allow to differentially identify the pool of genes specifically involved in the control of parasitaemia. Also, the kinetics of the package cell volume (PCV) should be monitored in order to collect blood samples for SAGE libraries at the precise time when the PCV increases as a result of efficient mechanisms of anaemia control. This would lead to the constitution of two global pools of genes involved in the trypanotolerance genetic character, either through the control of parasitemia and/or the control of anaemia, to set up field marker assisted selection and specific microarrays for further metabolic and pharmacological studies. Finally, these results could be compared with those of the QTL approach for cross validation and to identify positional candidate genes useful for future selection/introgression programmes in different cattle breeds.

The comparative SAGE libraries applied to the *Trypanosoma congolense* parasite should also allow for the identification of parasite genes that are specifically up- and/or down-regulated by the host defence mechanisms, with interesting consequences for drug development against animal and human trypanosomoses.

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