An integrated genomics approach towards analysis of resistance to mastitis in ruminants

R. Rupp[*], C. Bonnefont*[*†], D. Bergonier[†], M.R. Aurel [‡], P. Martin[#], P. Jacquiet[†], C. Moreno*, C. Robert-Granié*, P. Rainard[&] and G. Foucras[†]

Introduction

Amongst infectious diseases, mastitis is of major importance in dairy ruminants because of its high frequency and related costs. Causative micro organisms are mostly bacteria such as *Staphylococcus spp*., *Streptococcus spp*., or coliforms such as *E. coli*. Clinical mastitis is especially common in dairy cattle, with frequency of affected lactation per year ranging from 20 to 40%, and rather exceptional in dairy sheep and goat, e.g. less than 5% (Bergonier *et al.* 2003). Subclinical intra mammary infections, however, are widespread throughout dairy cattle sheep and goat herds.

Mastitis infections have important negative consequences on dairy ruminant health and welfare, and directly impact food safety. From an economical perspective, mastitis is associated with decreased milk production and additional costs related to increased culling rate, penalties on milk price, increasing treatment and waste of milk unfit for human consumption in clinical mastitis cases, and prevention. The average cumulated impact of mastitis in a French study involving 197 herds (1995-1997 figures) was 78 € per cow-year or 11€ per 1000 L of milk (Seegers *et al.* 2003). By extrapolation, the total annual costs for the French dairy cattle industry would have been about €250 million in 2005. In the UK, authors reported economic evaluations of the disease to be in the range from £106 million to £373 million per annum (Axford *et al.* 2000). Regarding dairy sheep and goat, the total EU milk production are 2.8 and 2 million tonnes, respectively. Making conservative assumptions of a 10% incidence of mastitis in the EU dairy sheep and goat flocks as a whole, giving a mean reduction in milk yield in affected animals of 20%, and a wholesale milk price of 650 (sheep) and 580 (goat) euros per tonne, the sole annual milk production losses to mastitis in small dairy ruminants can be estimated to be in the region of €60 million per annum. Davies *et al.* (2009) have placed mastitis as one of the major infectious disease in cattle and sheep, with respect to industry and public concern, economic impact, zoonotic potential and animal welfare, which is amenable to genetic selection.

[*] INRA, UR631 Station d'Amélioration Génétique des animaux, F-31326 Castanet-Tolosan, France
[†] INRA/ENVT, UMR1225 Interactions Hôtes-Agents Pathogènes, F-31076 Toulouse, France
[‡] INRA, UE0321 Domaine de La Fage, F-12250 Roquefort-sur-Soulzon, France
[#] INRA, UMR1313 Génétique Animale et Biologie Intégrative, F-78352 Jouy en Josas, France
[&] INRA, UR1282 Infectiologie Animale et Santé Publique, F-37380 NOUZILLY, France

Accumulating literature data over the last decades give strong evidence that the host's response protecting udder health is under genetic control in dairy ruminants. Up to date, evidence mainly arises from genetic parameters estimation for diseased status related phenotypes such as milk somatic cell counts (SCC) and clinical mastitis. Measured monthly, SCC can be interpreted as an effect of intra mammary infection and a good indirect indicator of mastitis. Heritability of SCC ranges from 0.11 to 0.18 in various sheep breeds and dairy breeds (see review by Mrode and Swanson 1996 and Heringstad *et al.* 2000).
Accordingly, many countries have updated their breeding objective and include mastitis resistance in cattle (Heringstad *et al.* 2000, Miglior *et al.* 2005) and sheep (Rupp *et al.* 2002). However, SCC-based or clinical mastitis-based selection requires large recording systems and adequate organisation at the population level. Favourable genetic decrease in clinical mastitis in Norvay (Heringstad *et al.* 2007) and Finland (Juga *et al.* 1999) give some evidence on the effectiveness of long-term selection for mastitis resistance. A Norwegian selection experiment (Heringstad *et al.* 2007) further added to the demonstration that considerable improvement can be achieved on mastitis resistance provided clinical mastitis data is used and sufficient selection intensity is applied. However, in most countries SCC is the only measure available. Additionally, SCC (and clinical mastitis) is (are) used as a phenotypic black box selection tool that might not fully consider the variety of pathogens responsible for mastitis and complexity of the resistance traits. Considerable improvement to genetic control of mastitis in dairy ruminants might therefore be gained from a better knowledge about genes and mechanisms underlying resistance to the disease.

The objective of the project, whose main results are reported here, is twofold: 1) to assess the consequences of SCC-based selection and 2) to develop a model that allows investigations of host mechanisms leading to improved resistance to mastitis and the knowledge of its genetic determinism. The project is based on a divergent selection experiment in dairy sheep and on multidisciplinary approaches including patho-physiology, immunology and genomics. It involved mainly four INRA research teams and experimental facilities that collaborated in the context of regional (Midi-Pyrénées) and national (ANR, Apisgene) grants as well as within the EU Network of Excellence EADGENE (http://www.eadgene.info/).

Divergent selection experiment

Creation of the High and Low SCS lines. The selection experiment was first described by Rupp *et al.* (2009). Briefly, a one-generation divergent selection of Lacaune dairy ewes based on estimated breeding values (EBV) for milk cell counts of dams and sires was initiated in 2003 at the INRA experimental facility of La Fage (UE321, Roquefort. France). Individual EBV based on the lactation mean of somatic cell scores (**SCS**), were obtained from the national genetic evaluation scheme implemented in the French Lacaune breed since 2002. Each year, two groups of about 6 progeny-tested rams, with extreme EBV for SCS were used to sire female offspring of 2 lines designated as Low SCS and High SCS lines. Rams were required to show similar and favourable EBV for milk production
From 2005 to 2009, a total of 504 ewes, sired by 68 different rams, started a first lactation at the INRA experimental facility, eg 238 in the High and 266 in the Low SCS line. Ewes in both lines were housed together in the same physical area and managed identically (machine-milked twice a day after the 28-day suckling period; diurnal pasture from April to August).

Observed divergence on somatic cell counts. Mean SCS of the High and Low SCS lines are in **Table 1**. The significance of the difference in milk somatic cell counts between the two lines was assessed by ANOVA with linear mixed models for test days SCS (about 9 records per lactation). Using 5214 test days SCS of the first three lactations of 392 ewes, ANOVA indicated a significant estimated effect ($P \leq 0.0001$) of line of 1.69 (±0.14) point on SCS. Previously published estimate based on 78 animals of the first cohort was similar, e.g. 1.69 (Rupp *et al.* 2009).

Resistance to intra mammary infections in the divergently selected SCS lines

Survey of natural infection in the experimental facility. Given the high prevalence of subclinical mastitis in commercial flocks, Low and High SCS animals reared at the experimental farm were considered to be equally and continuously exposed to mastitis pathogens. Resistance to natural infection was therefore assessed mainly by clinical survey of animals and repeated bacteriological analyses of udder-half milk samples.

Table 1: Somatic cell counts and mastitis frequency in the High and Low SCS line ewes

	High SCS line		Low SCS line	
	N	mean	N	mean
CCS (std), *10^6 cells/ml (715 L1-L3; 2005-2009)	2345	1193 (2904)	2869	277 (1298)
SCS (se), Lsmeans (715 L1-L3; 2005-2009)	2345	4,40 (0,13)	2869	2,70 (0,1)
Clinical mastitis (715 L1-L3; 2005-2009)		25 cases		6 cases
Mammary abscesses, % affected L1-L3 (2005-2008)	283	38%	???	7%
Milk Bacteriological Examination				
% positives, all samples[1]	1133	46%	1294	23%
% positives, at lambing	218	54%	248	22%

[1] sampling dates: lambing, suckling period, +3 additional dates between 2 month and 6 month DIM

Results from a 5-year survey period strongly suggest favorable responses to SCS-based selection on the resistance to natural intramammary infections. First, results suggest that Low SCS ewes were not at higher risk of clinical mastitis when compared to High SCS ewes. Indeed, although they were scarce, most clinical cases (n=25 out of 31, e.g average

frequency of 5%) occurred in the High SCS line (**Table 1**). Additionally, the incidence of chronic clinical mastitis, as detected by the systematic clinical evaluations of udders, was significantly higher in the High SCS than in the Low SCS line with 38% and 7% of ewes being affected, respectively (**Table 1**). The animals with abscesses were mostly ewes with long lasting intramammary infections, as evidenced by milk bacteriological examinations. Results were therefore in agreement with favorable genetic correlation between SCS and clinical mastitis that were reported earlier in cattle (Heringstad et al., 2003; Rupp and Boichard, 2003). They also support the hypothesis of a linear relationship between SCC and clinical mastitis, in the lowest values.

The analysis of instantaneous isolation results (**Table 1**) shows a significantly higher intra-mammary infection prevalence in High SCS than in Low SCS animals (OR=3.3 [2.5;4.4]), especially at lambing (OR=7.0 [4.2;11.7]). Comprehensive bacteriological analyses of the first cohort (9 sampling times and detailed identification of pathogens) gave further insight into the dynamics of infection (Rupp *et al.* 2009). The main conclusion was that duration of an infection was much higher in the High SCS line when compared to the Low SCS line (Rupp *et al.* 2009). Altogether, the results seem to indicate that there is a strong individual component in ability to avoid durable establishment of infections. Interestingly enough, part of the differences of susceptibility between Low and High SCS lines seemed to be emphasized at the peripartum period. This result correlates well with literature data on increased susceptibility of the mammary gland during the peripartum period (Burvenich *et al.* 2007).

Response to experimental challenge with staphylococci in the divergently selected SCS lines

Results from the survey were validated and extrapolated by experimental infections conducted at the Veterinary School of Toulouse. Those experiments, based on a small number of animals allowed evaluating the response of High and Low SCS lines under controlled condition of infection, e.g. nature and quantity of bacteria entering the teat canal and time of infection.

Two groups of seven ewes were successively challenged by intracisternal route with two different bacteria: *Staphylococcus epidermidis and Staphylococcus aureus* (Bonnefont *et al.* 2009). The latter two-pathogen species are main causes for intramammary infections in dairy ruminants. For both bacteria, induced mastitis were more severe in the High SCS group than in the Low SCS group, as measured by clinical signs, rectal temperature and presence of mammary abscesses. Furthermore, milk production losses were higher in the High SCS line (-70%) when compared to the Low SCS line (-30%) in a 10 days period following the challenge with *S. aureus*. Bacterial titre (**Figure 1**) in milk was significantly higher in the High SCS line when compared to the Low SCS line (T-test, $p < 0.001$ in *S. epidermidis* experiment and $p<0.05$ in *S. aureus* experiment). Altogether, experimental challenge experiments indicate that selection for decreased SCS is correlated with better ability to control the development of bacteria and to limit consequences of infection and inflammation.

Proteomic analyses of milk samples of high and Low SCS sheep upon challenge further indicated limited deterioration of milk secretion in the Low SCS line when compared to the high SCC line. Indeed, the high SCC line showed increased proteolysis probably due to the major milk indigenous protease (plasmin) and increased blood-milk barrier permeability as suggested by the increase in serum albumin (Bianchi *et al.* 2007). In addition, an early marker of mammary infection was identified: the acute phase protein Serum Amyloid A (SAA) that was visible 24 h after challenge (Bianchi *et al.* 2007). Differential analysis of minor whey proteins after challenge is in progress.

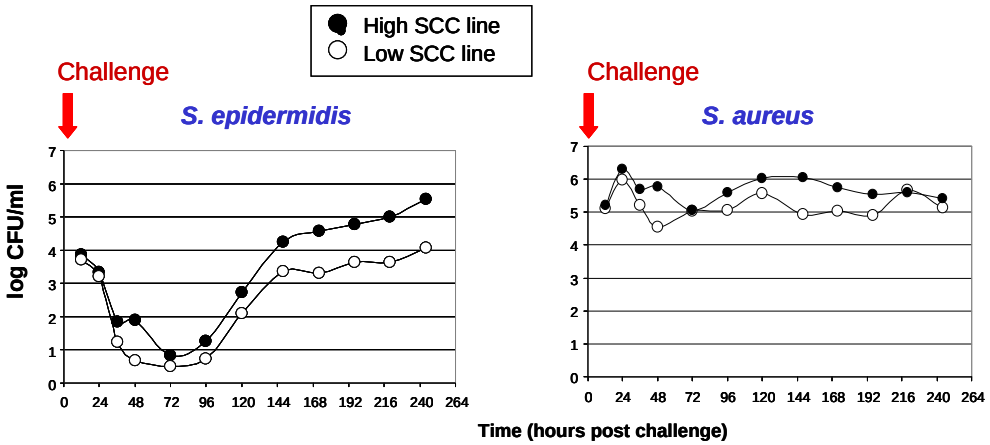

Figure 1: Bacterial titres after experimental challenge with *S. epidermidis and S. aureus* in High and Low SCS ewes.

Correlated response to infection with gastrointestinal nematodes in the divergently selected SCC lines

As the causative pathogens responsible for mastitis are mainly bacteria, the host's response to this disease is mediated by an adaptive immune response of inflammatory type (Th1/17). Conversely, the host's response to parasites such as nematodes is controlled by a type-2 immune response. From literature data, including several divergent selection experiments in mice (Mouton *et al.* 1984), poultry (Pinard-van der Laan 2002) and pigs (Crawley *et al.* 2005), there is accumulating evidence that different components of immune response, including type-1 and type-2 responses, and resistance to various diseases, were at least partially under independent genetic regulation. The question is raised, therefore, whether selection for improved resistance to mastitis has an adverse response on resistance to other diseases relevant to ruminant species, such as gastrointestinal parasitic infections (Davies *et al.* 2009).

To address that question, an experimental challenge experiment with *Haemonchus contortus* was implemented in two groups of 27 High and 26 Low SCC lambs (Traore *et al.* 2008). No significant difference was shown between the two lines of sheep for faecal egg excretions, worm's establishment and development, blood eosinophil counts, packed cell volumes, serum pepsinogen values and serum antibody measurements during three successive

experimental infections. Results therefore gave no indication of unfavourable effect of SCS based selection on resistance to parasite infections.

Transcriptomic analyses of the divergently selected SCS lines

Recent advances in microarray technology allow exploring expression of up to thousands of genes in the context of complex biological functions. Gene expression profiling is therefore a promising tool to contribute identifying genes products and pathways underlying mastitis resistance. We applied that technology in the context of the EU Network EADGENE mastitis group on the divergent line of sheep in order to provide insight into the genetically-determined differences in gene expression.

Gene expression profiling was applied to three major cell types which play a crucial role in host's response to intra mammary infections: milk cells, dendritic cells and mammary epithelial cells (Bonnefont *et al.* 2009). For analysing those cells, specific protocols for collection and/or stimulation were developed. Briefly, milk cells (mainly neutrophils) were collected in two groups of 6 Low SCS and 6 High SCS ewes, 12 hours after experimental challenge with Staphylococci bacteria. Dendritic cell progenitors were collected from bone marrow and differentiated in vitro from 4 Low SCS and 4 High SCS sheep. Dendritic cells were then stimulated by heat-killed *Staphylococcus aureus* which promotes DC activation/maturation. Mammary epithelial cells were isolated from two groups of six additional lactating primiparous ewes after culling. Microarray analyses were implemented using a 15K ovine oligonucleotide microarray (Agilent). Results gave insight into the major pathways and molecules activated in those cell types after challenge with *Staphylococcus sp.* For example, preliminary studies of mammary epithelial cells indicated the presence of several inflammatory mediators in supernatant (IL-8, CXCL3) after stimulation with bacterial products, showing that these cells may participate to immune responsiveness. Analyses of the SCS line effect in the three experiments are reported by Bonnefont *et al.* in the present WCGALP congress. Some commonalties were found (MAEPR1 gene, TLR signalling pathway) indicating that a few genes are consistently differentially expressed between resistant and susceptible animals, advocating their use for further investigation.

Towards the identification of genes underlying genetic resistance to mastitis

Interestingly, candidate gene studies for mastitis in dairy ruminants have been disappointing, with few validated and consistent gene effects, as for example the MHC alleles (Rupp and Boichard 2003). On the other hand, QTL detection studies have allowed localising regions of the genome that explain a large part of variability for udder health traits. In dairy cattle, two reviews (Khatkar *et al.* 2004; Smaragdov 2006) include around twenty references for QTL of mastitis resistance on almost all cattle autosomal chromosomes. To date, the only QTL fully characterized to our knowledge is the one on BTA22 (Sugimoto *et al.* 2006) where a polymorphism of the bovine forebrain embryonic zinc finger-like gene (FEZL) has been associated with its transcription activity, leading to control of cytokine expression and response to mastitis. Existence of QTL for mastitis resistance in sheep has also been

previously demonstrated in dairy sheep (Genesheepsafety EU project QLK5-CT-2000-00656). However, because of the low density of the microsatellite panel used at the time, results only gave limited resolution of the QTL locations and large chromosomal regions were not covered. Promising results are expected from the use of high-throughput SNP genotyping techniques and use of linkage disequilibrium to achieve very fine mapping and discover causal mutations.

In the context of a national project (SheepSNPQTL), funded by the French Research Agency (ANR) and APISGENE, we plan to fine map dairy sheep QTL for SCS using the Ovine SNP50 BeadChip (Illumina), driven by the collaborative efforts of the International Sheep Genome Consortium (ISGC) (www.sheephapmap.org). Two French ovine populations will be genotyped: i) a granddaughter design including about 1000 Lacaune and Manech AI rams and High and Low SCS ewes (about 200) that benefit from fine phenotyping in the experimental farm. Linkage analyses of the granddaughter population might therefore be completed by linkage disequilibrium analyses (in association with Linkage) in the divergently selected ewes and use of historical recombination events between the two connected populations. QTL detection will further benefit from an international collaborative genotyping effort (3SR EU project) of three French Spanish and Italian dairy sheep populations with SCC information. The project will then pursue fine mapping and genome mining of these sheep loci, to develop and verify causative polymorphisms or closely linked SNP markers suitable for use in selective breeding in several commercial dairy breeds. We expect that nomination of candidate genes will be greatly aided by functional genomic studies implemented in the high and low SCC lines of sheep.

Conclusion

A divergent selection experiment conducted in sheep for 7 years allowed to give strong evidence that SCS-based selection leads to decreased intra-mammary infection prevalence, clinical expression and associated mean SCS. Low SCS (resistant) animals showed improved ability to control bacteria multiplication. There was no evidence that SCS based selection will lead to modified resistance to gastrointestinal parasite infections. Transcriptomic profiling indicated some consistent differences between resistant and susceptible animals, providing information about genetically determined resistance mechanisms. Further studies will integrate both functional approaches and high throughput genotyping results.

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