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Selection for increased resistance to Salmonella carrier-state

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Improving the fowl's natural ability to clear *Salmonella* from their body is important in reducing disease prevalence in poultry flocks, as recommended by a recent regulation of the European Commission. It may be efficient, as expected from estimation of heritability coefficients : 0.16 in chicks and 0.18 for global contamination of hens. The animal's age has to be considered since the genetic correlation between resistances at the two ages is negative. Selecting two series of divergent lines for increased or decreased resistance, after inoculation at one week of age (chick resistance) or at the peak of lay (adult resistance) confirmed the efficiency at least of selection for the adult resistance. In parallel, genes controlling variations to *Salmonella* resistance were researched and several QTLs identified in crosses between experimental lines and, for some of them, confirmed in commercial lines. Thanks to the derivation of a model of *Salmonella* propagation within a flock, it has been shown that a combination of vaccination and genetic selection can result in very low percentage of contamination.

Keywords: Salmonella; genetic resistance; fowl; QTL; model

Introduction

In France as in other countries, *Salmonella* remains a major cause of human disease related to food consumption, and may result in mild to severe gastroenteritis, which can result in individuals with reactive arthritis or immuno-suppressions, in death (Velge *et al.*, 2005). Poultry products, especially eggs and egg products (EFSA, 2007b), are responsible for the majority of food-borne diseases, and the serovars *Salmonella enteritidis* and *Salmonella typhimurium* are the most often implicated, as reported to

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the Centres for Disease Control and Prevention in USA, from 1985 to 1999 (Patrick *et al.*, 2004). These *Salmonella spp.* serovars, like many others, may durably colonise the gastro intestinal tracks of chickens without resulting in any visible clinical signs of disease, which would help the farmer to identify contamination. This 'silent carrier' state will in turn lead to horizontal transmission within the flock after faecal shedding or, for *Salmonella enteritidis*, into vertical transmission though trans-ovarian route (Humphrey *et al.*, 1989).

These problems form the basis of a new regulation being implemented by the European Commission to reduce *Salmonella* prevalence in poultry flocks at less than 2% while the latter was recently estimated in EU at 29.7 and 23.7% of laying and broiler flocks respectively, with large differences between countries (EFSA, 2006; 2007a). Reduction of prevalence may be achieved by different prophylactic means, including vaccination (Zhang-Barber *et al.*, 1999), competitive exclusion (Rantala and Nurmi, 1973) and acidification of feed, but none of these precautions ensures zero risk of animal contamination and human transmission, at least when used alone. Improving genetic resistance to *Salmonella* would be a complementary way to reduce infection rates and propagation. The goal is to increase fowls' natural ability to clear *Salmonella* from their bodies. This may be evaluated by measuring the persistency of the bacterial infection after inoculation. To this end, many protocols of experimental infection have been developed, with different routes and doses of inoculation, time intervals between inoculation and assessment of bacterial contamination. Most of them consider the acute phase of infection, a few days after inoculation while some others, *e.g.* those developed by Duchet-Suchaux *et al.* (1995) in chicks and Protais *et al.* (1996) in adult hens, aims at reproducing the long term and asymptomatic carrier-state.

Using the latter models of infection, heritability of resistance has been estimated at 0.20 in young birds (Berthelot *et al.*, 1998) and higher than 0.35 in laying hens (Beaumont *et al.*, 1999), showing a partial genetic control of duration of *Salmonella* carrier-state. A selection experiment was initiated to test the feasibility of a genetic improvement of the ability of poultry to eliminate *Salmonella*, thereby reducing the risks of foodborne infections. In parallel, genes controlling variations to *Salmonella* resistance were researched and mathematical modelling of *Salmonella* propagation in laying hen flocks initiated.

The goal of this publication was to give some insights on the most important results of these studies.

Resistance

Initial experiments regarding resistance to disease were undertaken as early as in the 1930's, (Beaumont *et al.*, 2003a). The goal was to reduce the frequency of the disease and resulting mortality, at a time when many animals died from acute salmonellosis. No experimental inoculation was needed to observe diseased animals and experiments could be achieved directly on the field. The higher resistance of White Leghorns was then described by Robert and Card, quoted by Hutt and Scholes (1941). But De Volt *et al.* (1941) showed that the former were less resistant than selected Rhode Island Red hens; this result and others contributed to the development of selection for a higher resistance.

The first selection experiment for resistance to salmonellosis after deliberate inoculation was undertaken in 1932; and proved to be efficient. Indeed, heritability of reduced mortality was estimated at 0.15 by Beaumont *et al.* (1999) and at 0.12 by Janss and Bolder (2000), respectively, at day one and 14 of age, respectively. However, the latter authors also noticed that estimated genetic parameters strongly suggested that more

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resistant animals would survive longer and could thus contribute to a higher risk for consumers if they were still carriers of the bacteria.

Increased food safety can only result from a decreased risk for apparently healthy animals to carry bacteria (*i.e.* from an increase in genetic resistance to carrier-state). Since heritability estimates showed to be of moderate or even high value (Berthelot *et al.*, 1998; Beaumont *et al.*, 1999), an experiment of divergent selection was initiated by Beaumont *et al.* (2009). The base population was issued from a layer-type line and two series of divergent lines were selected, for increased or decreased resistance after inoculation at one week of age or at the peak of lay. These will are referred to as Saly+ and Saly- for the lines selected at a young age to increase (Saly+) or decrease (Saly-) the level of *Salmonella* contamination while Sala+ and Sala- are the names of lines selected at the adult age. Adult hens were orally contaminated and bacterial contamination of caeca, spleen, liver and ovary assessed four weeks later, as described in Protais *et al.* (1996). The selection criterion was an all-or-none trait called global contamination and coded '1' if at least one organ was found positive and '0' in the other cases. In chicks, resistance was assessed from the logarithm of the number of cfu. per gram of caeca (*i.e.* contamination level) five weeks after oral inoculation, as described in Duchet-Suchaux *et al.* (1995). A total of 3817 animals were thus measured; 1408 at the adult age and 2409 at the younger age (Beaumont *et al.*, 2009).

Most probably because the Saly+ and Saly- lines were only separated from the Sala+ and Sala- lines at the third generation, the difference between the Sala+ and Sala- lines remained small, *i.e.* from 2.19 in the Saly- to 2.56 log(cfu) in the Saly+ line. Larger differences were observed between the Sala- and Sala+ lines. At the 5th generation, average contamination levels were equal to 30 and 44% in the caeca, 5 and 6% in liver and 15 and 30% in spleen respectively, so that the mean percentages of global contamination ($adult_g$) were equal to 41 and 60% respectively. Selection may, therefore, be efficient in reducing the level of *Salmonella* carrier-state (Beaumont *et al.*, 2009)

This experiment allowed estimation using a large data set and thus with a good precision of parameters of genetic resistance and its links with production traits. Heritability of resistance was estimated at 0.16 in chicks and at 0.18 for global contamination of hens. In adult fowls, the genetic control appeared to be partly dependant on the organ. Heritability estimates varied from 0.14 to 0.23 and genetic correlations between contamination of the different organs ranged from 0.46 to 0.67. This result is in agreement with current biological knowledge on the differences between infection of systemic organs (spleen and liver) and intestinal tracts. Indeed, caecal contamination, which occurs earlier and for longer periods than systemic contamination (Duchet-Suchaux *et al.*, 1995), was the most heritable. On the whole, the positive values of estimated correlations were favourable for selection. Increasing resistance to global contamination should also decrease ovarian contamination, which is the major feature for the risk of human transmission but whose occurrence is too low for efficient selection. Global contamination of each animal appears to be the most efficient selection criteria. It allows the assessment of the carrier state more precisely through the results of three tests, thus reducing the risk of false negative results. Moreover, it combines several traits, which are positively correlated between each other. But, these results also underline that assessment of resistance depends on the organ in which *Salmonella spp* are searched for, which reinforces the importance of a very accurate definition of the resistance trait.

An important feature was the negative (-0.50) correlation between adult global contamination and chick caecal contamination. This negative relationship showed that increasing genetic resistance of hens will reduce resistance in chicks. Partially different

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genetic controls of adult and chicken resistance have been suspected, but not to such an extent. This result implies that results observed at one age may not be extrapolated to the other without experimental validation.

This study allowed estimating of genetic correlations between resistance and production traits. Most estimated genetic correlations were of moderate values, showing loose biological relations between resistance and production traits, suggesting that selection for an increased resistance should not have much effect on other traits, which is favourable. In particular, no clear putative effect of selection for increased laying rate on resistance could be observed. Negative correlations between egg colour and adult contamination were observed but must be confirmed.

Identification of marker genes for resistance

Although these results may seem promising, selection for an increased resistance would be very difficult to implement in breeder flocks, as they require experimental infections in protected areas, which are very expensive. Nevertheless, it should now be possible to alleviate the need of such measures by using molecular markers related to resistance. Genomic selection based on a large number of anonymous markers should also be very efficient on such traits (Goddard and Hayes, 2007)

Pioneer studies of Bumstead and Barrow (1988; 1993) examined the impact on inbred lines differing in resistance to mortality after experimental inoculation. Their higher genetic homogeneity increased the statistical power (*i.e.* the probability of detecting QTL or of evidencing differences of expression) of genomic studies, leading to the identification of QTLs for resistance to disease (Mariani *et al.*, 2001) and to their finer mapping (Fife *et al.*, 2009) as well as allowing testing candidate genes (Hu *et al.,* 1995), even if other crosses have been used since (Kramer *et al.*, 2003 or Lamont *et al.*, 2002).

Initial investigations have been made using candidate genes, *i.e.* genes chosen according to an *a priori* knowledge of their effect in *Salmonella* resistance. In particular, many studies focused on *SLC11A1* and *TLR4*, two genes known to be involved in resistance to *Salmonella* in mice. *SLC11A1* was first called *Nramp1* (natural resistance-associated macrophage protein) and shown to be responsible for the difference between mice strains in resistance to inoculation with *Salmonella typhimurium*, (Roy and Malo, 2002), *Mycobacterium bovis* and *Leishmania donovani*, because of its role in the control of the intracellular replication of parasites in phagosomes. After its identification, it was recognised as a member of the solute carrier family and renamed *Slc11a1* (Vidal *et al.*, 1993) The chicken homologue of *Nramp1* was mapped on the chicken chromosome 7 (Hu *et al.*, 1995) and subsequently cloned (Hu *et al.*, 1996). The second candidate gene, *TLR4* (Toll-like receptor 4) was previously named *Lps*, because its mutation results in a lack of response to LPS (lipo-polysaccharides specific of the Gram negative bacteria, including *Salmonella*) and a higher susceptibility to those bacteria. It was shown to belong to a family of innate immune system receptors (Toll-like receptors) and was mapped to the chicken micro chromosome 17 and cloned (Leveque *et al.*, 2003).

Many studies have dealt with these two genes. Both are involved in the early response of chicks to an acute infection with S. *typhimurium* (Hu *et al.*, 1997). *NRAMP1* was also shown to be involved in early stages of systemic *Salmonella* infection in meat-type chicks (Kramer *et al.*, 2003), in a cross between layer-type strains or breeds (Lamont *et al.*, 2002; Liu *et al.*, 2003) or in pullets (Girard-Santosuosso *et al.*, 2002). While a potential role of *Nramp1* in later stages of infection was also shown in mice inoculated

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with S. *enteritidis* (Caron *et al.*, 2002), it is of note that the *NRAMP1* allele coding for a better resistance to an early and acute infection was responsible for a higher excretion rate in later stages. While not yet investigated in fowls, this result implies, at least in mice, that animals selected for the *NRAMP1* allele coding for resistance to disease could be more susceptible to carrier-state, showing the importance of the choice of the selection criteria and the use of a relevant protocol of experimental inoculation. In hens, *NRAMP1* was shown to be associated with later spleen contamination of hens (Beaumont *et al.*, 2003b), while the role of *TLR4* could not be confirmed in the same study.

In addition to these two genes, several QTL detection programs were carried out in crosses between inbred lines differing for resistance to disease for a large range of *Salmonella* serotypes (Bumstead *et al.*, 1988; 1993) or to *Salmonella* carrier-state (Tilquin *et al.*, 2005). Several QTLs were identified (Mariani *et al.*, 2001, Tilquin *et al.*, 2005) in these genotypes. However, the latter study used selective genotypings (*i.e.* only those of the most extreme animals) which could have resulted in biases. Calenge *et al.* (2009) genotyped all animals in the formerly identified QTL regions, confirming two out of the four QTLs. The same authors tested the effect of the genome regions identified by Tilquin *et al.* (2005) in animals close to commercial ones. They used data and samples issued from the selection experiment and could thus confirm the role of the QTL on chromosome 1 and of *SLC11A1* in both chicken and laying hens. Development of a large scale arrays thanks to the identification of a large number of SNPs should soon result in the identification of new genome regions and in a shorter term, SNP-assisted or even genomic selection.

In parallel, differences in gene expression after a *S. enteritidis* infection were identified between poultry lines, either for candidate immune genes (Sadeyen *et al.*, 2004; 2006; Kaiser *et al.*, 2006), or using large scale arrays as in Van Hemert *et al.* (2006; 2007). These results contributed to the identification of the genes involved in resistance, provided that the mutations responsible for resistance result in differences in their expression levels.

In conclusion, many genome regions have already been identified regarding their effect on resistance to *Salmonella*, but most of them were only studied using one protocol of experimental inoculation (*i.e.* one serovar, one route of inoculation, one single animal age and genotype). These results cannot thus be extrapolated to other conditions without confirmation. Particular care must be taken regarding the negative genetic correlation between resistance of chicks and laying hens, which might lead to different strategies for layer- and meat-type lines.

Modelling

All the former results show the complexity of genetic control for resistance to disease carrier states. This first depends on the animal's age but also on the target organ, so that several selection criteria should be used to increase bacterial clearance. This choice is an important issue. The same holds true for the global strategy of genetic improvement. For instance, should the resistance of all animals be improved or would it be more efficient to increase it in smaller proportion of animals but to a larger extent? Answering such a question will clearly benefit from the comparison of the impact of these different options on the rate and levels of contamination of laying hens flocks. Modelling *Salmonella* propagation within a flock allows such comparisons. Prevost *et al.* (2006; 2008) derived mathematical models and, in addition to naïve (also called susceptible) birds, they distinguished three steps in the contamination:

- digestive contamination when only the digestive tract harbours *Salmonella spp.*

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- systemic infection (when systemic organs such as liver or spleen are contaminated after translocation of bacteria through the digestive barrier, which may also result in egg contamination) and
- bacterial clearance leading to recovery.

The development of the models and, in particular, the fitting of key parameters used in the results from the selection experiment, allowed identification of the most important factors for the risk of egg contamination and thus to human contamination risks. These included:

- the recovery rate is dependent on the ability of hens to eliminate *Salmonella.* This influences both the maximal prevalence and the duration of the epizooty.
- the rate of return to the susceptible state depends on the loss of protective immunity.

This modelling showed that the effect of selection of both parameters were not the same when selecting for a higher level of bacterial load than for a lower one: The recovery rate was altered when selecting for reduced carrier-state while the rate of return to the susceptible line was mainly responsible for the higher susceptibility of Sala+ lines. This result is in agreement with results on immune response of lines differing (among other traits) in resistance to carrier states, whether inbred (Sadeyen *et al.*, 2006), or outbred (Proux *et al.*, 2002; Protais *et al.*, 2003). Moreover, in the latter study, vaccine efficiency was found to be better in the more resistant lines. This difference in immune response also suggests the vaccines will be less efficient in lines more susceptible to carrier-state. This difference was found in simulations when comparing responses to selection in populations mimicking selected lines (Prevost *et al.*, 2008). It is important to notice that the combination of vaccination and genetic selection results in a very low percentage of contamination, very similar to the level the European community is requesting.

This study also compared the kinetics of infection between a homogenous population corresponding to the base population from which selection was undertaken, or a mixture of the Sala+ and Sala- lines with the same geometrical mean, which is the same duration of the different steps of infection. Introducing Sala+ animals reduces the peak of infection (*i.e.* the maximal percentage of infected animals), since accelerated bacterial clearance of Sala- animals reduces the risk of cross contamination. But it also delays the extinction of the epizooty, because Sala- animals require more time to recover. Inversely, results of genotype comparisons will not be the same when animals with different degree of bacterial clearance are reared together or not. This point should be further studied in practice.

These models may be further improved toward a finer modelling of the animal innate immune response which may allow the animal to get rid of any digestive contamination. Individual variations need to be more thoroughly investigated through individual-based models. This will allow consideration at the individual level and not, as previously, for the whole population, summarising it through the proportions of the different categories of animals (naïve, infective at the digestive level, systematically infected or recovered). Such investigations are currently underway.

Conclusions

Selection for a reduced propensity to carrier state of disease in poultry is possible and may synergise with other means of prevention. It will no doubt benefit from Marker

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Assisted or Genomic Selection. But, whatever the sophistication of the method of selection, its efficiency will be dependent on the choice of selection criteria. Large genotype x environment interactions influence resistance traits and special care must be taken of the animal's age. Conversely, literature results must be compared using a detailed definition, as underlined in general by Hughes *et al.* (2008) concerning animal trait ontology.

In parallel, lines differing in resistance will be useful as resources for research towards a deeper understanding of the genetic control of resistance and of the interaction between host resistance and pathogens.

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