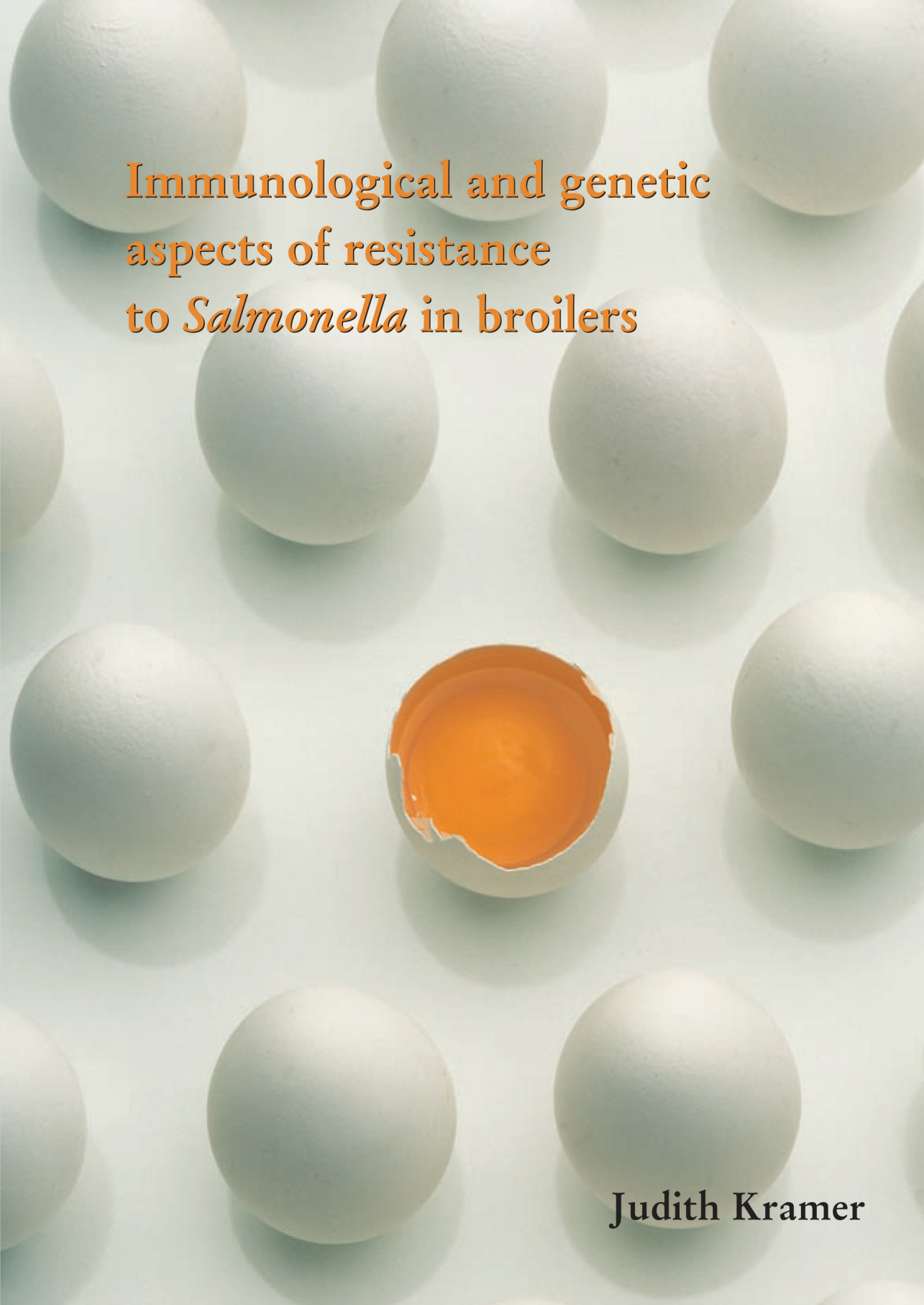
# Immunological and genetic aspects of resistance to *Salmonella* in broilers



Judith Kramer

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Immunologische en genetische aspecten van resistentie tegen *Salmonella* in vleeskuikens

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## Immunologische en genetische aspecten van resistentie tegen *Salmonella* in vleeskuikens

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# ABSTRACT

Salmonella can be a threat for both public health and economy. Salmonellosis is one of the most common food-borne diseases in human. The economical problems in the poultry industry are caused by treatment of the infection, and the infection might lead to high levels of mortality of the flock. Moreover, the infection might lead to decreased animal welfare. To prevent these infections, and their consequences in chicken, birds can be vaccinated or treated with antibiotics. However, the use of antibiotics and vaccines has been criticized because of the possible development of antibiotic resistant bacteria and the potential dangers of antibiotic and vaccine residues in animal-derived food products. A solution to those issues could be the enhancement of natural genetic resistance, by improving the reaction of the host to a pathogen. This can be accomplished e.g. when the immune response of poultry can be enhanced. Research described in this thesis, provides insight on the differences in immune responses between chickens and within chicken lines in relation to disease resistance. Results of in vitro and in vivo studies showed, that the innate immune response played an important role in chickens in relation to natural and disease resistance. Furthermore, several genes were identified that were associated with Salmonella resistance in chicken. Overall, this study confirmed that disease resistance to Salmonella is a polygenic trait, which makes it difficult to apply in breeding strategies. However, together with other strategies to control Salmonella that already exist, the genetic approach may provide additional means to reduce the transmission of the infection and control Salmonella.

# WOORD VOORAF

Zo, daar is mijn boekje dan. Dit werk is natuurlijk geworden zoals het is met behulp van vele anderen. Hiervoor wil ik een aantal mensen graag bedanken, hoewel woorden niet alles kunnen zeggen.

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# CONTENTS


| | | |
|---|---|---|
| CHAPTER 1 | General introduction | 11 |
| CHAPTER 2 | Entry and survival of *Salmonella* enterica serotype Enteritidis PT 4 in chicken macrophage and lymphocyte cell lines | 27 |
| CHAPTER 3 | Macrophage activity early after infection with *Salmonella* Enteritidis | 39 |
| CHAPTER 4 | Comparison of natural resistance in seven genetic groups of meat-type chicken | 49 |
| CHAPTER 5 | Characterization of the innate and adaptive immunity to *Salmonella* Enteritidis PT 4 infection in four broiler lines | 67 |
| CHAPTER 6 | Genetic resistance to *Salmonella* Enteritidis carrier-state of five different genetic groups of meat-type chicken | 83 |
| CHAPTER 7.1 | Association of twelve candidate gene polymorphisms and response to challenge with *Salmonella* Enteritidis in poultry | 97 |
| CHAPTER 7.2 | Differential gene expression in cell lines after *Salmonella* inoculation | 113 |
| CHAPTER 8 | General discussion | 121 |
| SUMMARY | | 129 |
| NEDERLANDSE SAMENVATTING | | 133 |
| REFERENCES | | 139 |
| PUBLICATIONS | | 157 |
| CURRICULUM VITAE | | 159 |

# Chapter 1

## GENERAL INTRODUCTION

## *SALMONELLA*, THE BACTERIUM

Gaertner is credited for the first isolation of *Salmonella* in 1888. The term *Salmonella* was only adopted many years later in honor of Daniel. E. Salmon, who was involved in early *Salmonella* studies.

*Salmonella*e are members of the family *Enterobacteriaceae*, and are gram negative, facultative anaerobic, non-sporogenic, usually flagellated (with a few exceptions, though) rods. DNA hybridization studies showed that the genus *Salmonella* consists of only two species, *Salmonella* enterica and *S.* Bongori (Le Minor and Popoff, 1987). *S.* Enterica is distributed into seven subspecies. The first subspecies that belongs to group 1, named *S.* Enterica subspecies Enterica, infects warm-blooded animals (including human). The other groups affect cold-blooded animals, and rarely infect man (Humphrey et al., 1998). In total, over 2,200 serotypes of *Salmonella* are currently identified (Le Minor and Popoff, 1988).

*Salmonella* bacteria show considerable antigenic diversity. White (1929) developed a scheme which was modified by Kauffmann (Kauffmann-White scheme (Popoff et al., 2001)), based on antigenic structure, whereby strains could be identified by serotyping. In this scheme, each *Salmonella* serotype is recognized by its possession of a particular lipopolysaccharide (LPS) or O-antigen and a flagellar or H-antigen. These antigens form the major components of the bacterial surface.

Furthermore, phage typing is widely used for subtyping of certain *Salmonella* serotypes. Phage types are generally considered to be stable and definitive epidemiological markers. For *S.* Enteritidis in Europe (and in the Netherlands), phage type (PT) 4 is the most prevalent phage type (Pohl et al., 1991; Schroeter et al., 1994; Nastasi and Mammina, 1996; RIVM, 1996-2002; Cieslik et al., 2001), whereas in the USA PT8 an PT13a are the most frequently isolated phage types (Hickmanbrenner et al., 1991; Usera et al., 1994; Liebana et al., 2002).

## SALMONELLOSIS

Various *Salmonella* strains produce a wide variety of infections. Onset of symptoms occurs 6 to 48 hours after ingestion of *Salmonella*. Typhoid serotypes such as *S.* Typhi in humans and *S.* Gallinarum in chickens can produce typhoid like symptoms, such as sustained fever, headache, malaise, loss of appetite, abdominal pain and enlargement of the liver and spleen, severe systemic infections. Most *Salmonella* serotypes are non-typhoid and cause gastro-enteritis in man and animals involving nausea, vomiting, abdominal cramps, diarrhea, fever, and headache. Both man and animals can also be carrier of the bacteria without having any visible clinical symptoms. Various serotypes can cause disease in multiple species (like *S.* Enteritis or *S.* Typhimurium), others show a more restricted host

specificity, like *S.* Typhi and *S.* Paratyphi which is strongly associated with disease in humans and primates, *S.* Gallinarum and *S.* Pullorum which are strongly associated with infections in poultry and *S.* Choleraesuis which predominantly infects pigs.

*Salmonella infections in mammals*

In the eighties of the last century in most European countries, the number of *Salmonella* cases in humans caused by *S.* Enteritidis increased enormously (van Pelt et al., 1999a). Different phage types of this serotype have replaced *S.* Typhimurium as the most frequently isolated serotype. Also in the Netherlands the number of human *Salmonella* cases caused by *S.* Enteritidis increased between 1987 and 1994, relative to other *Salmonella* bacteria. Thereafter, it appears that the percentage of the number of cases decreased slowly (Figure 1.1; RIVM, 1996-2002).

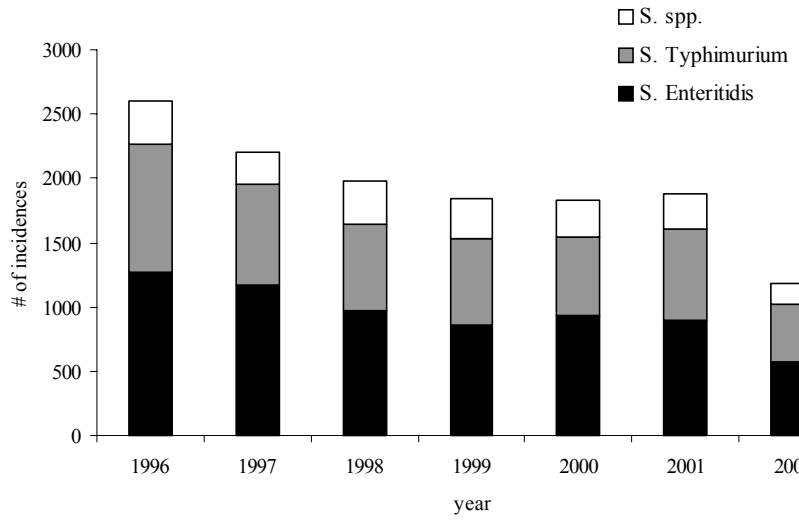


**Figure 1.1.** *Salmonella* serotype number and distribution of isolates from The Netherlands. For 2002 only until week 44 the numbers are calculated (RIVM, 1996-2002).

In the Netherlands, 2,089 *Salmonella* strains from human cases have been sent to the National Institute of Public Health and the Environment in 2001 (RIVM, 1996-2002). This number is much lower than the number of actual cases, since only a limited number of people ask for medical attention, the causative agent is not isolated or serotyping is not requested. It is estimated that each year about 50,000 people become infected with *Salmonella*. In contrast to other western countries, in the Netherlands the number of cases

confirmed in the laboratory decreases gradually since the mid eighties (van Pelt et al., 1999a).

Outbreaks of salmonellosis in man are mainly caused by poultry food products (21% of the cases caused by chicken and 39% caused by eggs), but also pork and beef are an important source of *Salmonella* infections (respectively 26% and 10%; van Pelt et al., 1999b). Especially young children and elderly people are susceptible for a *Salmonella* infection. The main factors that contribute to the outbreaks are poor temperature control in preparing, cooking, and storing food (Passaro et al., 1996; Todd, 1997).

*Salmonella Enteritidis in poultry*

Pathogenesis

The pathogenicity of *Salmonella* depends on the bacterial serotype and strain, the age of the birds, the genetic background of the host and the portal of entry (Barrow, 2000). *S.* Enteritidis is capable of producing a typhoid-like disease in very young chicks. Most strains of *S.* Enteritidis contain a large plasmid encoding a number of virulence genes including a cluster of genes (named spv genes; Gulig et al., 1993) whose exact function is unknown, but which is essential for the invasion of the bacteria in liver and spleen, producing a systemic disease (Barrow, 2000). In hens, which are in lay, sometimes the reproductive tract becomes infected, particularly the ovary and the oviduct (Hopper and Mawer, 1988; Humphrey et al., 1989; Barrow and Lovell, 1991; Baskerville et al., 1992; Keller et al., 1995). This might result in an infection of the egg by *S.* Enteritidis thus causing vertical transmission. Egg contamination may also occur by faecal surface contamination of the shell at the time of lay (Barrow and Lovell, 1991). Adult birds are relatively resistant to *Salmonella*, although *Salmonella* will colonize in the intestine or cause a systemic infection, in the absence of disease. Infection of adult birds results in faecal shedding which is much lower than after infection of young chicks, that results in faecal excretion of high numbers of *Salmonella* for several weeks. It is supposed that due to the presence of a complex intestinal flora in adult birds, the multiplication of *Salmonella* in the intestinal flora is less extensive in adult birds (Barrow, 2000).

*Salmonella* infections usually start after oral ingestion followed by colonization in the gut, which usually results in extensive shedding in the faeces. Bacterial entry is frequently initiated by adhesion to epithelial cells of the mucosa, where after the intracellular bacteria invade the host through the epithelial layers or lymphoid tissue, including the Peyer's patches, (Neutra and Kraehenbuhl, 1992; Zhang-Barber et al., 1999). In mice and humans, it has been shown that *Salmonella* penetrates the mucosal epithelium of the small intestine using M cells (Neutra and Kraehenbuhl, 1992). The mechanism whereby *Salmonella* migrates from the submucosa to other organs in chickens is unknown. A lot of bacteria may be removed by non-specific defense mechanisms such as gut peristalsis, or

may be destroyed by professional phagocytes without necessitating the specific attention of the immune system. Bacteria that survive these non-specific defense reactions colonize deeper tissue sites and stably infect a suitable niche. At this stage, the host generally pays sufficient attention to the infectious agent and tries to eliminate the infection as indicated by the development of an immune response. However, *S.* Enteritidis is able to survive and replicate within various leukocytes, like macrophages and heterophils (Stabler et al., 1994). Hence, leukocytes can play an important role in the dissemination of *Salmonella* from the gut to various organs, such as liver and spleen (Popiel and Turnbull, 1985).

Finally, it is possible that, although the *Salmonella* infection is undetectable in the living birds, the bird is still a carrier of *Salmonella* (Qin et al., 1995; Desmidt et al., 1997). Then the infection will become apparent, when through stress or concurrent diseases the immune response of the chicken is lowered.

Epidemiology

There are many sources of *Salmonella* infection for poultry, although the major sources are limited to the animals themselves, feed and the environment. Chickens become infected with *Salmonella* either by vertical or horizontal transmission. With vertical transmission the breeder flock is infected with *Salmonella* through which the egg becomes infected by the parent. This can be due to an infection in the female genital tract before the eggshell is formed. Additionally, more often this can be due to contaminated faeces adhered to the eggshell in the cloaca, and the bacteria enters the egg through the shell when cooling down after lay.

After hatching, it is possible that the chick becomes infected through horizontal transmission. This route of infection is not derived from the parent, but can be derived from any other route of contact of the pathogen with the chick. Usually a bird becomes orally infected by eating or picking *Salmonella* contaminated materials, like faeces, dust, or feed, or via other contaminated birds.

Along with the increase of the number of *S.* Enteritidis infections in man, a dramatic increase in the isolation of *S.* Enteritidis has been observed world-wide in livestock poultry and poultry products. Also in the Netherlands the number of *S.* Typhimurium infections decreased, and the presence of *S.* Enteritidis increased dramatically in both man and chickens between 1987 and 1994. After than, the number of human cases decreased gradually (van Pelt et al., 1998; 1999a).

Caecal carriage of *Salmonella* in chickens was measured in the Netherlands for one year from March 1992 to March 1993 by Jacobs-Reitsma and coworkers. In this study, 27% of the broilers flocks were infected with *Salmonella* at slaughter (Jacobs-Reitsma et al., 1994). In 1997 in the Netherlands, an eradication program was started to decrease the number of contaminated flocks. From that time, the number of infected flocks (determined

in caecum) decreased approximately from 28% to 12%, measured in June 2001 (Dellaert, 2001). Hopefully, the eradication program will continue to decrease the percentage of infected flocks the following years.

Additionally, in the Netherlands, *S.* Paratyphi B variation Java increased in broilers from less than 2% of all isolates before 1996 up to 40% in 2001. However, although the exposure of humans to contaminated poultry meat is relatively high, human patients with a *S.* Paratyphi B variation Java infection are still rare (van Pelt et al., 2002).

*Salmonella*e have been shown to be very persistent in the environment. They are capable of surviving more than one year in poultry houses, despite cleansing and disinfecting, over two years in artificially contaminated poultry meal (Davies and Wray, 1996) and over 2.5 years in avian faeces (Morse et al., 1978). However, the presence of *Salmonella* in the environment does not always result in infection of the flock. This seems to be related to the number of organisms present and the opportunities for multiplication, spread, and ingestion. Animal related factors are stress and concurrent diseases during exposure to environmental contamination with *Salmonella* (Bailey, 1993; Davies and Wray, 1996; Kinde et al., 1996).

Immunology

The immune response can be divided in two different responses, innate immunity and adaptive immunity. The early phases of a response, without prior exposure, depend on the innate immunity, whereas the adaptive immunity depends on specific responses of antigen specific lymphocytes, which are developed upon and after exposure to an agent. The adaptive immune response can be classified into humoral or antibody mediated and cell mediated immunity. After an initial infection, the response occurs in three phases (Figure 1.2).

The innate immune system is capable of removing the infectious agent shortly after the infection through direct killing of the pathogen, and to activate and regulate immune reactions of both innate and adaptive immune system by antigen presentation and production of effector molecules.

Two major components of the innate immune system will be activated to dispose the infection, humoral factors and phagocytosis. These humoral factors, like lysozyme, acute phase proteins, increase in concentration in response to an infection. In addition complement will be activated, which is involved in mediating adherence between complement-coated microorganisms and phagocytes. These adherence reactions result in enhanced phagocytosis and killing of the organism or in exocytosis of cell contents that will kill the bacteria (Powell, 1987). Polymorphonuclear leukocytes (PMN) are a group of cells mainly involved in phagocytosis and intracellular killing of pathogens. The phagocytic heterophils (the equivalent of the neutrophil in man) and macrophages (mature monocytes) are thought to be

the most important cell types involved in early inflammation after infection with *Salmonella* in chicken (Powell, 1987).

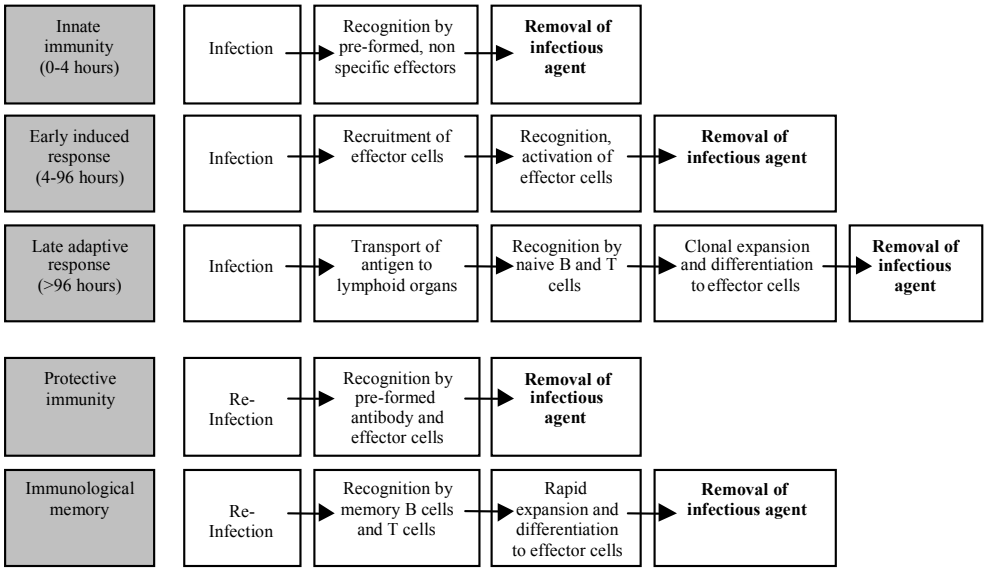


**Figure 1.2.** The response to an initial infection occurs in three phases (Janeway and Travers, 1997). The effector mechanisms that remove the infectious agent (eg phagocytes, NK cells, complement) are similar or identical in each phase but the recognition mechanisms differ. Adaptive immunity occurs late, because rare, antigen-specific cells must undergo clonal expansion before they can differentiate into effector cells. The response to re-infection is much more rapid ; pre-formed antibodies and effector cells act immediately on the pathogen, and immunological memory speeds a renewed adaptive response.

The interaction between *Salmonella* and the epithelium triggers the chemotaxis of phagocytic cells to the site of infection (Desmidt et al., 1997; Henderson et al., 1999). Chemotaxis of these phagocytic cells will stimulate respiratory burst, caused by a variety of stimuli, like phagocytosis of particles or interaction of membrane receptor with chemoattractants (Babior, 1984). The respiratory burst results in the production of radicals, like superoxide, hydrogen peroxide and nitric oxide. These radicals are important in the microbicidal activity, but can also result in significant oxidative damage of tissue in localized areas of inflammation (Dietert et al., 1991; Coleman, 2001). Stimulation of phagocytes that occurs in early stages of inflammatory response results in the production and secretion of monokines, like interleukine (IL)-1, IL-2, IL-6, and tumor necrosis factor (TNF)-α (Bombara and Taylor, 1991; Qureshi et al., 1994; Rautenschlein et al., 1999; Kaiser et al., 2000). Finally, actual phagocytosis of bacteria is an important activity of the phagocytes for various processes, like clearance of the pathogens, internalization of antigens

for subsequent processing and presentation and for destruction of the pathogen within the phagocytes (Dietert et al., 1991).

After entrance of *Salmonella*, phagocytic cells will start to eliminate the pathogen, whereby the adaptive immune response, both humoral and cell-mediated immunity, will be activated. Usually a *S.* Enteritidis infection will result in the production of antibodies of all three isotypes, IgM, IgG and IgA by plasma cells in the intestinal secretion, bile, blood, and egg yolk (Humphrey et al., 1991; Barrow, 1992; Desmidt et al., 1996). These antibodies will opsonize the pathogen, through which a link is provided between the microorganisms and phagocytes (Powell, 1987). As is the general rule for most antigens, IgM is produced first in response to infection with *S.* Enteritidis, followed by IgG. Serum IgG can be detected from one to two weeks onwards after an experimental infection with *S.* Enteritidis, then IgG titers rise to reach peak values between 2 and 3 weeks after infection. Thereafter the serum titers remain high for at least several months post-infection (Chart et al., 1990; Barrow and Lovell, 1991; Chart et al., 1992). The magnitude and the speed of the induction of serum IgM and IgG after an experimental infection is directly related to the inoculum dose and age (Humphrey et al., 1991; Chart et al., 1992; Kramer et al., 2001). Desmidt and colleagues indicated that elimination of *S.* Enteritidis from chickens partly depends on humoral immunity, whereby the intestinal humoral response appeared more effective than the systemic humoral response for elimination of *S.* Enteritidis (Desmidt et al., 1998). Moreover, Arnold and Holt (1995) also described an increase in intestinal shedding of *S.* Enteritidis after suppression of the humoral response.

In the latter study by Arnold and Holt (1995), the role of the cell-mediated immune response by T-cell depletion was also investigated. This also resulted in an increase in intestinal shedding. Although T-cells do not interact with microbes directly, these cells interact with other cell types of the infected host. Moreover, T-cells produce interferon (IFN)-γ, through which the phagocytes will be activated. Thus immunocompetence of a chick appeared to rely on interdependent functions of multiple components of the immune response, i.e., aspects of both humoral and cell-mediated immunity (Arnold and Holt, 1995).

## CONTROL OF *SALMONELLA*

Because of the zoonotic aspects of *Salmonella* (the economic losses due to medication and hospitalization of humans, and the economical problem for poultry farmers) it is important to control, and if possible, eradicate *Salmonella* from the poultry flocks. Therefor, several control strategies have been developed to prevent infections.

*Hygiene and management*

Chickens can easily become infected by *Salmonella* in the hatchery and during rearing. This infection can be acquired from feed, rodents, wild birds, insects, fomites and farm personnel. It is possible to rear poultry that are free of the various serotypes of *Salmonella*, but this requires high costs for housing, and tight control on feed quality, hygiene and management. Management schemes for staff and entry of personnel, like restricted entry, appropriate clothing, and washing and cleaning of both staff and clothing are important to prevent infection (Barrow, 2000). After an infected flock has been removed, thorough sanitation is needed, by cleansing and disinfecting the house and its environment to prevent re-infection of subsequent flocks (Davies and Wray, 1995).

Furthermore, hygiene during processing should also be an important issue to prevent *Salmonella* infected poultry products. Modern poultry processing implies a slaughter capacity of more than 6000 birds per hour and this can only be realized with complete mechanized and automated processing lines. The removal of the gut including caecum during this automated process is a risk, because these organs are easily ruptured, where after the *Salmonella* infected contents can contaminate the processing line. It is possible to slaughter the *Salmonella* negative flocks first, before the *Salmonella* positive flocks. To control *Salmonella* during processing it is also important to control the hygienic conditions of catching, transport and holding at the slaughter plant. Stress can also result in increased excretion of pathogenic microorganisms of chickens that were already infected. Furthermore, it is important to clean and disinfect the transport crates, containers, and vehicles after transport to control the infection (Mulder, 1998).

Thus although it is possible to prevent *Salmonella* infection by hygiene and management, these procedures are not always economically feasible and it requires strict rules to prevent infection throughout the whole process.

*Antibiotics*

Antibiotics are commonly used to control or prevent several bacterial infections like *Salmonella* in poultry. In addition, antibiotics have been advocated for controlling *Salmonella* carriage by laying hens. However, antibiotic therapy in food production animals is increasingly coming under close inspection, mainly because of the development of antibiotic resistant bacteria and increased susceptibility to infections (Barrow, 2000; Hernandez et al., 2002). Additionally, depending on the choice of the agent, antibiotics may not always be effective to eliminate *Salmonella* (Smith and Tucker, 1975). Furthermore, after withdrawal of the antibiotics there may be a short period when the birds may become unusually susceptible to *Salmonella* infection, largely because normal flora, itself inhibitory to *Salmonella*, is also affected by antibiotic usage (Barrow, 1997).

*Vaccination*

One of the aims of vaccination is to prevent infection, replication and spreading in flocks. Vaccines should induce a strong protective immunity against re-infection of the host. Nevertheless, results of the use of vaccines based on killed bacteria were variable, because birds were only partially protected. Shedding was reduced to certain extent in faeces (Barbour et al., 1993; Nakamura et al., 1994b), or the protection lasted only for a few weeks (Gast et al., 1992; Gast et al., 1993).

Over the years, life attenuated strains have shown to be more effective compared to killed vaccines (Barrow, 1991; Griffin, 1991; Lillehoj, 1991; Cooper, 1994; Barrow, 1996). This is probably due to the stimulation of both cellular and humoral responses by life vaccines while killed vaccines only stimulate humoral responses (Chatfield et al., 1993; Barrow, 1996). Several groups have investigated the use of attenuated mutant strains of *Salmonella* as life vaccines (Barrow, 1990; Hassan et al., 1991; Cooper, 1994; Cooper et al., 1994; Cooper et al., 1996; Curtiss and Hassan, 1996). So far, most life attenuated vaccine strains reduce faecal shedding after challenge with the wild strain, and some vaccines limit or prevent organ invasion from the gut (Cooper, 1994; Hassan and Curtiss, 1997; Dueger et al., 2001; Feberwee et al., 2001). Sometimes, immunization with a life vaccine induces cross-protective immunity against various *Salmonella* serovars (Curtiss and Hassan, 1996; Hassan and Curtiss, 1997; Dueger et al., 2001). Vertical transmission can also be reduced by vaccination of adult animals. In addition, the immunity can be transmitted through the egg to the newly hatched chick. Maternal antibodies can achieve a reduced intestinal colonization and shedding as well as a reduction or prevention of internal organ invasion after infection with wild type *Salmonella* strains (Hassan and Curtiss, 1996). On the other hand, these maternal antibodies may also decrease the effect of vaccination of the progeny, thus additional vaccination at 2 and 4 weeks of age is necessary (Hassan and Curtiss, 1996). However despite these successes, vaccines are mostly quite expensive. Moreover, the safety of life *S.* Enteritidis vaccines is still uncertain because it might be pathogenic to human, although it is not pathogenic to poultry. This makes it difficult to apply in the poultry industry.

*Competitive exclusion*

The mechanisms used by one species of bacteria to exclude or reduce the growth of other species are various, but Rolfe (1991) determined that there are at least four major mechanisms of competitive exclusion. These mechanisms are: 1) creation of micro-ecology that is hostile to other bacterial species, 2) elimination of available bacterial receptor sites, 3) production and secretion of antimicrobial metabolites, and 4) selective and competitive depletion of essential nutrients (Rolfe, 1991).

At hatch, most chicks have very little microflora in their gut and they are highly susceptible to intestinal colonization by *Salmonella* (Pivnick and Nurmi, 1982). At two weeks of age, chicks have a mature gut flora (Barnes et al., 1972) and are therefore much more resistant to intestinal colonization. Increased resistance of an adult animal can be transferred to the newly hatched chick by oral treatment with cultures of the caecal flora, as first reported by Nurmi and Rantala (1973). Since then, the efficacy of competitive exclusion to prevent colonization of pathogenic bacteria in chicks has been demonstrated by many others (Snoeyenbos et al., 1978; Barnes et al., 1980; Pivnick et al., 1981; Bailey, 1988; Bailey et al., 1988; Impey and Mead, 1989; Hinton et al., 1990; Hume et al., 1998). The disadvantage of using undefined cultures is the inadvertent introduction into stock of other pathogens with the preparation. Because of this, undefined cultures are not acceptable by the regulation authorities in a number of countries (Stavric, 1992; Stavric and Daoust, 1993). However, results of searches for individual protective strains were only partially successful compared to the undefined cultures. For example the use of various strains of Lactobacilli as a probiotic in poultry resulted in reduced *Salmonella* colonization (Edens et al., 1997; Pascual et al., 1999). Amongst many other strains from different genera tested for their inhibitory effect on *Salmonella* colonization, *Salmonella* strains themselves were found to be most inhibitory. Therefore, attenuated *Salmonella* vaccine strains were used (Berchieri and Barrow, 1990; Methner et al., 1997; Methner et al., 1999) or other, non-pathogenic *Salmonella* strains were used to prevent colonization by exclusion effect (Barrow et al., 1987; Martin et al., 1996).

*Consumer education*

The incidence of human infections by *S.* Enteritidis could be reduced when the general public realizes that eggs are raw food. The use of raw eggs in foodproducts has contributed directly to foodborne illness. Poultry meat also needs to be thoroughly heated, to kill the bacteria present on the meat. In addition, after preparing poultry meat, it often happens that *e.g.* the plates or knives used were not thoroughly cleaned where after an infection can occur when these materials are used again. Thus, to decrease human cases of salmonellosis, it is important to increase the public awareness of the hazard of consuming poultry products (www.alimentationinfo.org; www.cdc.gov/foodsafety/diseasebac.htm).

*Genetic resistance to Salmonella*

Differences in susceptibility between and within lines in mammals and poultry

A number of studies have indicated that there is considerable variation in different chicken lines in the response to viral, bacterial and parasitic pathogens. Natural disease resistance refers to the inherent capacity of an animal to resist disease when exposed to pathogens, without prior exposure or immunization (Hutt, 1958). The earliest examples of

differences in susceptibility of chickens to bacterial infections are described in the forties and fifties, to *S.* Pullorum and *S.* Gallinarum (Hutt and Scholes, 1941; Smith, 1956). More recently, Bumstead and Barrow (1988; 1993) reported that inbred lines of white leghorn chickens showed large differences in susceptibility to different serotypes of *Salmonella* (*S.* Typhimurium, *S.* Gallinarum, *S.* Pullorum, and *S.* Enteritidis). It appeared that the same lines were resistant or susceptible to the different serotypes of *Salmonella*. Thus these results suggest that there may be a general mechanism of resistance that may apply to all serotypes of *Salmonella* in chickens (Bumstead and Barrow, 1988; Bumstead and Barrow, 1993). Furthermore, other studies also described differences in susceptibility between various chicken lines in response to *Salmonella* Enteritidis (Guillot et al., 1995; Protais et al., 1996; Duchet Suchaux et al., 1997; Girard Santosuosso et al., 1998; Kramer et al., 2001).

Some of the observed variation in natural resistance is related to age, infection dose and route, environmental factors (*e.g.* stress) and food intake (Bumstead and Barrow, 1988; Nisbet et al., 1994; Klasing, 1998; Kramer et al., 2001). But a significant component of variation in natural disease resistance and susceptibility to *Salmonella* infection appears to be heritable and therefore to be passed stably from parent to offspring (Berthelot et al., 1998).

Candidate genes

Genetic resistance to *Salmonella* or other pathogens may be associated with a single gene but it is usually associated with multiple genes. In the last few years, genes have been found that strongly influence the level of host resistance and several of these have been identified using inbred strains of mice. Candidate genes involved in natural resistance to various *Salmonella* strains in mice are natural resistance macrophage protein 1 (*NRAMP1*, formerly *Ity/Lsh/Bcg*; Blackwell et al., 1994; Skamene, 1994; Blackwell, 1996), inducible nitric oxide production (*iNOS*; Barrera et al., 1994), Lps gene locus (O'Brien et al., 1980; O'Brien et al., 1985), MHC molecules (Soo et al., 1998) or Toll Like Receptor (*TLR*) 5 (Sebastiani et al., 2000).

Clear genetic differences in the susceptibility of chickens to infection by *Salmonella* have been observed and it has been possible to identify relatively more resistant and susceptible lines of chickens. Although not as many genes have been identified in chicken as in mouse, several candidate genes or regions have been described for chicken. *NRAMP1* and TNC (a locus closely linked to the *LPS* gene in the mouse genome) showed a significant association on early resistance to *Salmonella* (Hu et al. 1997; Liu et al., 2002). Another region that was identified on chicken chromosome 5, designated *SAL1*, showed a large effect on resistance to *Salmonella* (Mariani et al. 2001). Cotter et al. (1998) showed that the B-complex determined part of the differential resistance to *S.* Enteritidis. Furthermore, Liu and Lamont (2003) described associations on early resistance to

*Salmonella* in chicks with inhibitor of apoptosis protein 1 (*IAP-1*), prosaposin (*PSAP*) and caspase I.

Differential gene expression

Differences in levels of gene expression following invasion of *S.* Enteritidis might provide insight into mechanisms of immunopathogenesis of salmonellosis in chicken. Furthermore, differences in expression levels might explain some variation in *Salmonella* resistance that is present between and within lines. Differential cytokine expression in avian cells was found in response to various *Salmonella* serotypes, *e.g. S.* Enteritidis (Kaiser et al., 2000). After infection with *S.* Enteritidis, no differences in response were found on the production of *IFNγ* compared to non-infected cells. Bao et al. (2000) also showed that *IFNγ* is plays a critical role in intestinal immunity against *Salmonella*. However, a slight downregulation of *IL-1* and *IL-2* mRNA levels and an eight-fold increase of *IL-6* was found in infected cells compared to non-infected cells (Kaiser et al., 2000). In another study, differences in *iNOS* expression and activity was found between two different chicken strains, a hyper- and a hypo-responder (Dil and Qureshi, 2002).

In the future, the use of gene array technology for gene expression profiling will become more and more common. Rosenberger et al. (2000) reported a study of the use of gene array technology to determine genes involved in host responses to *S.* Typhimurium in mice. After stimulation of macrophage cells with LPS or infection with *S.* Typhimurium, several differences in gene expression were found compared to non-stimulated or non-infected cells. The expression of genes like tristetraprolin (*TTP*), *iNOS*, the chemokines *MIP-1α* and *MIP-1β*, *IL-1β*, *CD40* and *TGFβ2* and others were induced by *S.* Typhimurium and LPS. On the other hand, other genes like cyclin D1, E and F, Ski and IL-6 receptor β were down regulated. Gene expression profiles promise to yield insight into complex interactions between host and bacterial gene expression (Rosenberger et al., 2000; Rosenberger et al., 2001).

## APPLICATIONS

This overview described and discussed various aspects involved in a *Salmonella* infection, and various ways to prevent or control the infection. All control strategies have their positive prospects, but most of them also have certain prospects that make them unfavorable to use to control infections. As described above *e.g.*, the use of antibiotics will be restricted in the future, and hygiene and management is possible but it is difficult and costly. Vint (1997) proposed that changes in the virulence of pathogens, concentration of poultry in larger production units, and failure of pathogen eradication in most commercial operations require genetic approaches to improve disease resistance. Genetic improvements

of disease resistance can increase immune responses, and will reduce the use of antibiotics and drug residues in food products, and therefore will increase animal welfare. Thus, selective breeding for genetic resistance offers an additional possible control measure to reduce *Salmonella*.

Different factors can be involved in disease resistance according to the interaction of the host with a pathogen like *Salmonella*. When a pathogen enters the host, *Salmonella* is able to adhere to the intestinal wall where after invasion through the intestinal wall takes place. During these processes, the immune system plays a important role, and will be activated when *Salmonella* enters the host. All these factors and others are relevant, and play an important role in disease resistance. By concentrating on the immunological aspects of chickens to *Salmonella* infections, we have provided information and insight about the differences in immune responses between chickens and chicken lines in relation to disease resistance.

## INTRODUCTION TO PAPERS

The ultimate objective of this thesis was to develop genetic markers for macrophage functions and their role in resistance to *Salmonella* infection in chicken. There are a lot of indications that macrophages (or phagocytes), and the capability of *Salmonella* to survive intracellularly in phagocytes, play an important role in the pathogenesis and genetic resistance to *Salmonella*.

To confirm the important role of phagocytes during a *Salmonella* infection, in chapter 2 the differences in entry and survival of *Salmonella* between various leukocytes are described, using chicken macrophage and lymphocyte cell lines.

In chapter 3 the activity of macrophages early after an *in vivo* infection with *Salmonella* in chickens is described. Therefore, several assays were performed to characterize the activity and numbers of macrophages isolated form tissues that are involved in *Salmonella* infection.

Chapter 4 deals with an animal experiment, which was conducted to investigate whether differences exist in natural resistance between seven different meat-type chicken lines. Therefore several immune parameters of both innate and adaptive immunity were measured without a previous infection to measure the differences between lines.

Chapter 5 describes the characterization of the innate and adaptive immune responses after a *Salmonella* infection of 4 broiler lines. In this experiment, the colonization of *Salmonella* in various organs and different immune responses were measured after infection, to investigate if the differences in genetic resistance to *Salmonella* could be explained by differences in immune responses.

Then, an infection experiment was done (chapter 6) with five of the same chicken lines described in chapter 3, to determine if the differences in natural resistance between lines could explain the differences in genetic resistance to *Salmonella*. Therefore, chickens were infected with *Salmonella*, and the differences in levels of colonization between lines were determined early after infection.

Results and DNA derived from the experiment described in chapter 6, were used to study twelve candidate genes, as described in Chapter 7. In this study single nucleotide polymorphisms were investigated using the technique of polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Results indicated that all genes showed an association (significant or indication) between the various genotypes of the genes and the *Salmonella* load in at least one of the organs.

The general discussion, chapter 8, deals with the future prospects of genetic resistance to *Salmonella* in broilers.

# Chapter 2

## ENTRY AND SURVIVAL OF *SALMONELLA ENTERICA* SEROTYPE ENTERITIDIS PT4 IN CHICKEN MACROPHAGE AND LYMPHOCYTE CELL LINES

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**ABSTRACT**

Various leukocytes are involved in the reaction to counter *Salmonella* infection in chicken. The various leukocyte types react differently after an infection, since some clear the infection while others may cause dissemination of *Salmonella* throughout the chicken. Therefore, we investigated *in vitro* the entry and survival of *Salmonella enterica* serotype Enteritidis in chicken cell lines of various cell types, including two macrophage cell lines, HD11and MQ-NCSU (NCSU), two B-cell lines LSCC-1104-X5 (1104) and LSCC-RP9 (RP9), and a T-cell line MDCC-MSB-1 (MSB-1). The macrophages were able to internalize high numbers of *S.* Enteritidis. In contrast and as expected, cells of the T- cell line MSB-1 and the B-cell line RP9 internalized bacteria at a much lower level. After *S.* Enteritidis entered the macrophages, the number of intracellular *S.* Enteritidis decreased over time, so that after 48 hours no more than 20% of the bacteria, which had entered, survived intracellularly. In contrast to macrophages, the number of *S.* Enteritidis in cells of the T-cell line MSB-1 and the B-cell line RP9 increased rapidly within 12 hours post-inoculation. Thereafter the number of intracellular *S.* Enteritidis decreased only slowly. In conclusion, all three different cell types were able to control and to start clearing *S.* Enteritidis, although macrophages were far more effective compared to T- and B-cells. However, none of the cell lines were able to clear *S.* Enteritidis fully within 48 h. These results suggest that the three cell types play an important but different role in the dissemination and elimination of *S.* Enteritidis throughout the animal.

## INTRODUCTION

*Salmonella enterica* serotypes are facultative intracellular bacteria that include serotypes that are highly pathogenic for both humans and animals. Several serotypes are food borne pathogens and are zoonotic (*S.* enteritidis, *S.* Typhimurium). Other serotypes are primary pathogens (*S.* Gallinarum, *S.* Pullorum) for poultry and are important from an economic point of view. In different hosts, the different serotypes of *Salmonella* may cause diseases that vary from mild diarrhea to severe systemic infection (Finlay and Falkow, 1989; Barrow, 2000). The use of antibiotics and live-attenuated vaccines to control *Salmonella* infection in chickens has been criticized because of the possible development of antibiotic resistant bacteria, the potential dangers of antibiotic residues and because of residual attenuated vaccine strains in animal-derived food products for human consumption. Despite the use of antibiotics and vaccination, *Salmonella* infections are still widespread in poultry. Therefore, another alternative approach to control *Salmonella* infections in poultry is the enhancement of natural genetic resistance to *Salmonella*.

An infection with *Salmonella* usually starts by ingestion, followed by colonization in the intestine. After colonization, *Salmonella* is able to penetrate the mucosal epithelium, which results in a systemic infection of, *e.g.* spleen and liver (Desmidt et al., 1997; Henderson et al., 1999; Bao et al., 2000). Following infection, phagocytes start to eliminate *Salmonella* and subsequently stimulate T- and B-cells. Thus, both the non-specific (phagocytes) and the specific (T- and B-cells) immune responses are activated and play an important role in the host (Bloom and Boedeker, 1996; Kramer et al., 2001).

Adherence and entrance of *Salmonella* into an eukaryotic cell is thought to be a passive process or to be actively induced by the bacterium itself. The passive process is a cell receptor-mediated process due to the adherence of *Salmonella* directly to the cell receptor. Adherence to the cell receptor induces phagocytosis of the pathogen, but only professional phagocytes can efficiently perform this process (Schaible et al., 1999). The alternative mechanism, the active process by the bacterium, succeeds via host ligands present on the bacterial surface, which will result in invasion by *Salmonella*. *Salmonella* is able to induce extensive membrane ruffling of the host cell surface and by means of this membrane ruffling it is subsequently enclosed and taken up into the host cell (Francis et al., 1993; Joiner, 1997; Ernst et al., 1999; Schaible et al., 1999).

Because *Salmonella* are able to survive and replicate extracellularly and intracellularly *in vivo*, it has been suggested in mice that phagocytes (*e.g.* macrophages) may act as carriers transporting intracellularly residing bacteria throughout the host (Carter and Collins, 1974). Using human cell lines, survival of *Salmonella* was also described within T- and B-cell lines (Verjans et al., 1994). Thus in humans not only macrophages but also

T- and B- cells may play a role in the dissemination of *Salmonella* throughout the body (Verjans et al., 1994).

To investigate the putative role of the various cell types in the dissemination of *Salmonella* in chickens, we carried out experiments to investigate both the passive and active entrance and survival of *S.* Enteritidis PT4 within various chicken cell lines. We investigated two macrophage cell lines, two B-cell lines and one T-cell line.

## MATERIALS AND METHODS

*Cell lines*

Five different cell lines, two macrophage-like cell lines, two B-cell lines and one T-cell line were used for the entry and survival assays (kindly obtained from various non-commercial sources). The macrophage-like cell line HD11 was developed by viral transformation with myelocytomatosis virus MC-29 of chicken bone marrow cells (Beug et al., 1979). The macrophage-like mononuclear cell line MQ-NCSU (NCSU), was selected from the spleen of chicken challenged with the JM/102W strain of Marek's disease virus (Qureshi et al., 1990). The B-cell line LSCC-1104-X5 (1104) is a plastic-adhering lymphoblastoid chicken cell line, obtained from an early tumor of the bursa of Fabricius of a chicken infected with subgroup A of avian leukosis virus (Hihara et al., 1974). The B-cell line LSCC-RP9 (RP9) is a lymphoblastoid cell line. It was selected from the lymphoid leukosis tumor transplant LSCT-RP6 (Okazaki et al., 1980). The T-cell line MDCC-MSB-1 (MSB-1) was obtained from a splenic lymphoma of chicks infected with Marek's disease (Akiyama and Kato, 1974).

*Culture conditions*

Cells were grown in RPMI 1640 medium Dutch Modification (GibcoBRL, Life technologies, Paisley, Scotland) supplemented with 10% heat-inactivated fetal calf serum (FCS, ICN Biomedicals, Zoetermeer, The Netherlands), 50 IU/ml penicillin (ICN Biomedicals), 50 µg/ml streptomycin (ICN Biomedicals), 2mM L-glutamine (ICN Biomedicals), $5 \times 10^{-5}$ M 2-mercaptoethanol at 37ºC under 5% $CO_2$ and 100% humidity ($CO_2$ incubator). All cell lines were routinely grown in 175 $cm^2$ culture flasks and subcultured three times a week. For entrance and survival assays, $2 \times 10^5$ cells of each cell line were seeded in 24-well plates and incubated overnight in a $CO_2$ incubator using 1 ml culture medium without antibiotics. This resulted in about $6 \times 10^5$ cells per well, and the adherence of the HD11, NCSU and 1104 cell lines to the culture flasks.

*Bacterial strains and growth conditions*

A nalidixic acid resistant strain of *S. enterica* serotype Enteritidis PT4 was used (Van Zijderveld et al., 1992). This strain is pathogenic for humans, and it may cause clinical disease especially in young or weak chicks. For the cell culture inoculation experiments, fresh overnight cultures of *S.* Enteritidis were prepared by growing the bacteria in LB broth (Biotrading, Mijdrecht, The Netherlands). The bacterial culture was incubated at 37ºC under vigorous shaking (100 shakes per minute).

For the entry and survival assay, exponentially growing cultures of *S.* Enteritidis were prepared by diluting overnight cultures 1:100 in prewarmed RPMI 1640 medium and then incubating them for 3 hours at 37ºC under vigorous shaking, resulting in a culture of $7 \times 10^7$ colony forming units (CFU)/ml (determined by colony counting).

*Entry and survival assay*

The entry and survival assay was performed as described previously by Verjans et al. (1994), with a few modifications. Briefly, 0.4 ml of a fresh 3 hours *S.* Enteritidis culture ($2.8 \times 10^7$ CFU) was added to each well of the 24-well plates seeded with 1 ml cells, resulting in a nominal multiplicity of infection of approximately 70 (MOI=70). Adhesion and subsequent entrance of bacteria in cells was allowed to proceed for 30 minutes at 37ºC in a $CO_2$ incubator. At this point, gentamicin was added to a concentration of 200 µg/ml to kill extracellular bacteria, while the viability of intracellular bacteria was not affected (Vaudaux and Waldvogel, 1979). To confirm that all extracellular *Salmonella* were killed by gentamicin treatment, samples of the culture medium were taken after gentamicin treatment at 1 hour and 4 hours post-inoculation an plated on Brilliant Green Agar plates (BGA plates, Biotrading) with 100 ppm nalidixic acid. Then the cells were washed twice with 1 ml PBS supplemented with 5% FCS (washing buffer) to remove the gentamicin, cells were cultured in 1 ml RPMI 1640 medium supplemented with 10% FCS and 20 µg/ml gentamicin. The cells were cultured in medium with gentamicin to prevent re-infection with *S.* Enteritidis and to prevent the unlimited growth of *S.* Enteritidis in the medium that were derived from the release of *S.* Enteritidis from dead cells. At various time points (1, 4, 12, 24 and 48 hours post-inoculation), the number of intracellular *S.* Enteritidis per cell line was determined in triplicate, and each of the triplicates was plated in duplicate. Thus of each point, six measurements were taken.

The number of viable cells was determined by tryphan blue exclusion. The number of bacteria that entered the cells was adjusted to the number of viable cells of each cell line. During the experiment the number of cells increased per well for the T-cell lines (about nine times) and B-cell lines (about three times) but the number of cells for the macrophage cell lines remained more or less stable.

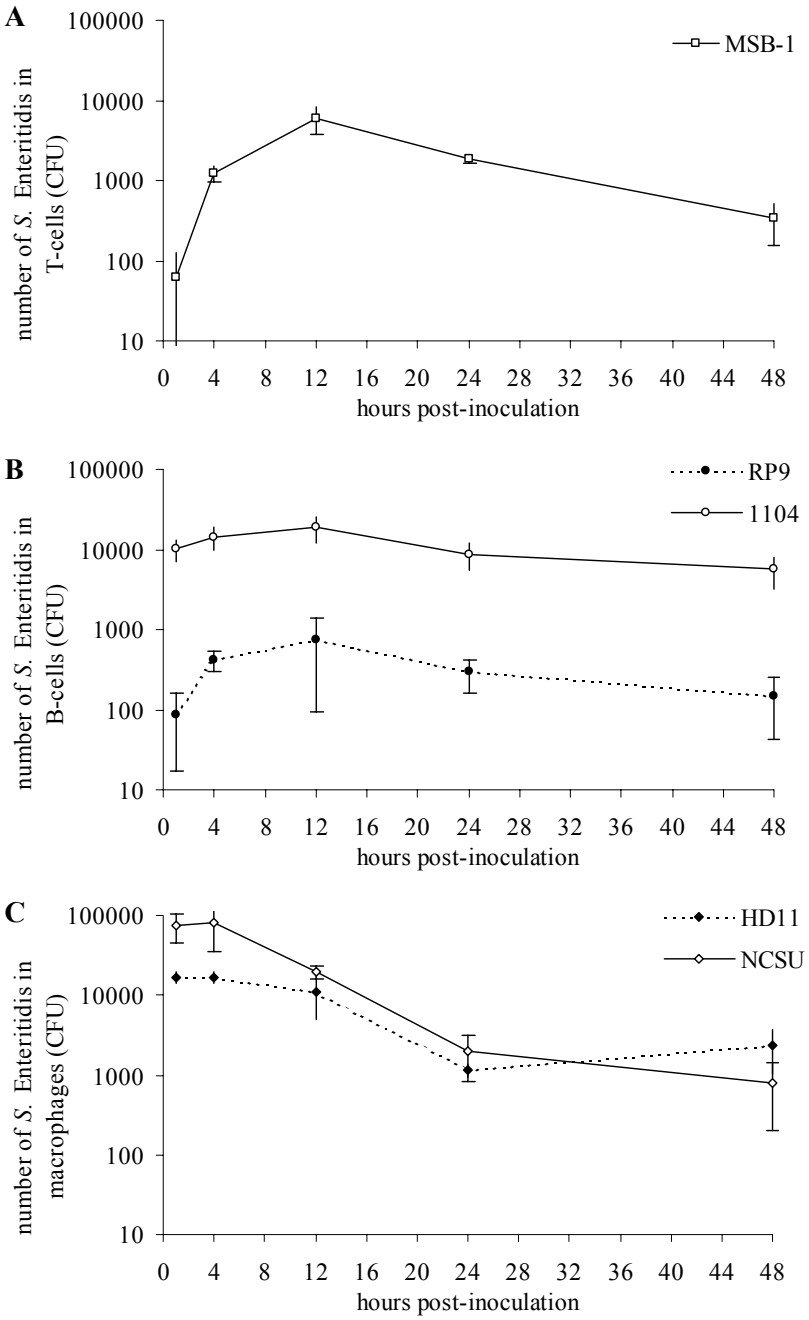


**Figure 2.1.** Bacterial growth kinetics within different chicken cell lines: a T-cell line MSB-1 (A); B-cell lines RP9 and 1104 (B); and macrophage cell lines HD11 and NCSU (C). Triplicate cell cultures were incubated with *S.* Enteritidis at a MOI of 70. Less than 1% of these bacteria entered the cells. Data presented are the average number of intracellular bacteria of six samples plus standard deviation. Asterisk (*) represents significant difference ($P < 0.05$) between two cell lines within one graph at a specific timepoint.

To determine the number of intracellular *S.* Enteritidis, cells were washed twice using washing buffer to remove the gentamicin. Subsequently, the cells were lysed by adding 1 ml PBS plus 0.2% (w/v) saponin to each sample and incubating the lysate for 5 minutes at room temperature to release intracellular *Salmonella*. Appropriate dilutions in PBS were plated onto BGA plates to determine the number of viable intracellular *Salmonella* (CFU) by colony counting.

This experiment was repeated three times, by which the exact number of bacteria in the 3 hours *Salmonella* culture varied between experiments, resulting in variable numbers of *Salmonella* entering the cells. Since the slope of the curves of survival in each cell line was similar for all three experiments, results of only one representative experiment are shown. In the experiment demonstrated, all cell lines were inoculated at the same time with the same 3h *Salmonella* culture. This allowed us to compare the different patterns of the different cell lines.

*Data analysis*

Comparisons were made between the different cell lines at each timepoint after inoculation. Moreover, comparisons were made between the different timepoints within one cell line, to study the kinetics of the graphs. The significance of differences between groups was analyzed by Student's t-test.

## RESULTS

*Entry and survival in T-cells*

The results of the entry and survival of *S.* Enteritidis into the T-cell line MSB-1 are shown in Table 2.1 and Figure 2.1A. Less than 1% of the bacteria in the inoculum entered the cell lines (Table 2.1). Entrance of *S.* Enteritidis in the T-cells occurred with the lowest frequency of the five cell lines tested (Table 2.1). Following internalization, at 12 hours the number of intracellular *S.* Enteritidis in the MSB-1 T-cell line increased significantly almost 100 times ($P < 0.01$). After this increase, the number of intracellular *S.* Enteritidis in the T-cells slowly decreased ($P < 0.01$) to a level, 48 hours post-inoculation, that was still more than 5 times higher than the number of bacteria that initially entered the T-cells ($P < 0.05$).

*Entry and survival in B-cells*

The kinetics of entry and survival of *S.* Enteritidis in the B-cell line RP9 (Figure 2.1B) was similar to that of the T-cell line MSB-1 (Table 2.1 and Figure 2.1B). At 12 hours post-inoculation the number of intracellular *Salmonella* increased almost nine times (Table 2.1, $P < 0.05$). Between 12 and 48 hours post-inoculation the RP9 B-cell line was

able to decrease the number of intracellular bacteria ($P = 0.06$). Despite the control of *Salmonella* from 12 hours on, after 48 hours the number of intracellular bacteria tended to be higher compared to the number of bacteria that entered the B-cells.

The number of *Salmonella* that entered the B-cell line 1104 was considerably higher than in the B-cell line RP9 ($P < 0.001$, Table 2.1 and Figure 2.1B). The number of intracellular bacteria in the B-cell line 1104 increased slightly from 1 to 12 hours post-inoculation ($P < 0.01$) and between 12 and 48 hours post-inoculation the number of *S.* Enteritidis somewhat decreased (up to 55% of the initially internalized bacteria).

**Table 2.1.** The absolute number of bacteria that entered and that survived in the cells after inoculation[a], and the bacteria / viable cell ratio (in parentheses)

| Cell line | Hours post-inoculation | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | | 4 | | 12 | | 24 | | 48 | |
| MSB-1 | 63 | (0.0005) | 1,248 | (0.01) | 6,050 | (0.01) | 1,888 | (0.002) | 335 | (0.0003) |
| RP9 | 88 | (0.0004) | 420 | (0.002) | 738 | (0.001) | 295 | (0.0004) | 148 | (0.0002) |
| 1104 | 10,125 | (0.04) | 14,500 | (0.06) | 19,000 | (0.06) | 8,775 | (0.02) | 5,600 | (0.007) |
| HD11 | 16,875 | (0.04) | 16,775 | (0.04) | 11,025 | (0.03) | 1,163 | (0.003) | 2,385 | (0.01) |
| NCSU | 75,000 | (0.5) | 82,500 | (0.5) | 19,875 | (0.1) | 1,990 | (0.01) | 808 | (0.004) |

[a]Inoculation with $2.8 \times 10^7$ cfu *S.* Enteritidis.

*Entry and survival in macrophages*

Results of the entry and survival of *S.* Enteritidis of two macrophage cell lines are shown in Table 2.1 and Figure 2.1C. Compared to the other cell lines, a considerable number of *S.* Enteritidis entered the macrophages ($P < 0.01$). The number of bacteria that entered the NCSU cell line was four times higher than in the HD11 cell line ($P < 0.01$). Between 1 and 4 hours post-inoculation the number of intracellular *S.* Enteritidis remained stable in both lines (see Table 2.1, bacteria / viable cell ratio). From 4 hours on, the number of intracellular bacteria that survived decreased in both cell lines ($P < 0.07$). The relative number of *S.* Enteritidis that survived was lower in the NCSU cell line between 4 and 12 hours post-inoculation compared to the HD11 cell line ($P < 0.05$). Between 12 and 24 hours post-inoculation, both cell lines showed the same decline in the curve, which demonstrates that the killing activity of *S.* Enteritidis in this timespan was equal in both cell lines. At 24 hours of inoculation, the number of intracellular *S.* Enteritidis remained more or less stable.

Finally, 48 hours after inoculation the NCSU cell line killed more than 95% and the HD11 cell line killed more than 80% of *S.* Enteritidis that had entered the cells.

## DISCUSSION

Marked differences were found in the initial uptake of *S.* Enteritidis between the three different cell types analyzed: T-, B-cells and macrophages. Overall, the five cell lines appeared to have a mechanism that limits survival of intracellular *S.* Enteritidis and an activity through which they decrease the number of intracellular *S.* Enteritidis. Nevertheless, all cell lines reacted differently to *S.* Enteritidis.

The two macrophage cell lines differed in the initial load of *S.* Enteritidis. The results indicate that the initial killing activity of *S.* Enteritidis between 1 and 12 hours post-inoculation by the NCSU cell line was more effective compared to the HD11 cell line. These differences in kinetics may be due to the origin of the cells or due to the viruses used to immortalize these cell lines. The HD11 cell line is originally derived from bone marrow cells infected by myelocytomatosis virus, whereas the NCSU cell line is originated from spleen cells infected by Marek's virus (Qureshi et al., 1990). It seems possible that macrophages isolated from the spleen (NCSU cells) are more mature and therefore better equipped to internalize bacteria than early and undifferentiated macrophages isolated from the bone marrow (HD11 cells; Beug et al., 1979). Another possibility that might influence killing is the differences in genetic background of the chickens used to prepare these cell lines. HD11 cells were generated using SPAFAS chicks (Beug et al., 1979) while NCSU cells were generated using Dekalb XL female chicks (Qureshi et al., 1990). These differences in genetic background of the chickens, together with the origin of the cells, may have contributed to the observed differences in kinetics.

Differences in uptake ($P < 0.05$) and survival ($P < 0.05$) of *S.* Enteritidis were also seen between the T- and B-cell lines (Figure 2.1A and B). Overall the bacterial load in 1104 B-cells remained more or less stable during the whole experiment and was higher than that in the RP9 B-cell line and the MSB-1 T-cell line ($P < 0.05$). After an increase of the number of intracellular *S.* Enteritidis from 1 to 12 hours upon inoculation of the RP9 B-cell line and the MSB-1 T-cell line, these cell lines were able to control, but not to clear fully *S.* Enteritidis within 48 h. Verjans et al. (1994) also described differences in the entrance of *Salmonella* (*S.* Typhimurium) in a T- and a B-cell line. Although these latter cell lines were of human origin, the authors also described that the number of *Salmonella* that entered B-cells was higher than the number that entered to T-cells and none of the cell lines was able to clear *Salmonella* within 24 h. In contrast to our results, Verjans et al. (1994) showed that upon entry, *Salmonella* was not able to replicate extensively within the cells, while in our results the number of intracellular bacteria both in the B-cell line RP9 and in the T-cell line

MSB-1 increased ($P < 0.05$). These differences may be due to the origin of the cells. The cell lines were derived from different species (human versus chicken), they were cultured from different organs and were immortalized using different viruses. Moreover, different *Salmonella* strains were used for the inoculation (*S.* Typhimurium versus *S.* Enteritidis). Furthermore, Verjans et al. (1994) also showed that differences occur in adherence and invasion between different bacterial strains. In this study, we were did not determine the number of bacteria that adhered to the cells. However, while optimizing the procedure, immunohistochemical staining was used to determine whether the bacteria adhered and actually entered the cells, these results did not show large differences between the five cell lines (unpublished results). Generally, these results show that in both T- and B-cell lines *Salmonella* can enter and survive in the cells, whereby the B-cell lines seemed to be more susceptible to *Salmonella*.

In this study, the number of *S.* Enteritidis that initially entered the macrophage cell lines was higher compared to the number of *S.* Enteritidis that entered the lymphocytic cell lines ($P < 0.01$). The high numbers of *S.* Enteritidis that entered the macrophages can be explained by phagocytosis of *S.* Enteritidis by the macrophage cell lines and by invasion of the pathogen in these cells. In contrast, *Salmonella* present in T- and B-cell lines is probably only due to invasion (Ernst et al., 1999; Schaible et al., 1999). These results show that also in chickens two independent mechanisms may exist for infection of the cell by *Salmonella*, namely phagocytosis (passively for *Salmonella*) and invasion (actively by *Salmonella*).

Not only was the initial uptake of *S.* Enteritidis by macrophages the highest of all cell lines, but these lines also started clearing *S.* Enteritidis immediately after inoculation (the bacteria / viable cell ratio decreased from 4 hours on). In contrast, the T- and B-cells were not able to control the intracellular bacteria immediately after *S.* Enteritidis entered the cells. Although it may also reflect a selective preference of the bacterium, these results indicate that macrophages are professional phagocytes by the high number of *S.* Enteritidis that had entered the cells and macrophages are able to start killing *S.* Enteritidis immediately after inoculation.

Overall, after 24 hours of inoculation, all cell lines became more or less stable in their bacterial load. From that time, the number of intracellular bacteria decreased only slightly and no cell lines were able to fully clear *S.* Enteritidis at 48 h. These results suggest that after *Salmonella* enters the leukocytes, these cell types have the potential to become a carrier of *Salmonella in vivo*.

When a chicken becomes infected with *Salmonella*, the bacterium passes the intestinal epithelium. Most likely the macrophages that are present in the lamina propia of the gut will internalize *Salmonella* very effectively and become infected, but also the T- and B-cells present are susceptible and can become infected with *Salmonella*. These results indicate that not only macrophages fail to prevent a systemic infection by the dissemination

of the bacteria in macrophages to other tissues (Carter and Collins, 1974), but also the T- and B-cells might play an important role in the dissemination of *Salmonella* in the body. The pathogenesis of *Salmonella* and the cell types that play a role in the dissemination of *Salmonella* causing a systemic disease are two aspects that need to be investigated more thoroughly to understand salmonellosis in chicken.

# Chapter 3

## MACROPHAGE ACTIVITY EARLY AFTER INFECTION WITH *SALMONELLA* ENTERITIDIS

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**SUMMARY**

To investigate the role of macrophages early after *Salmonella* infection, groups of five 4-week-old white Leghorns were orally infected with $10^9$ cfu *S.* Enteritidis. At 0, 24, 48, and 72 hours after infection, spleen, ileum, and caeca were removed and used for isolation of viable cells. Bacteriological examination demonstrated that oral infection led to high numbers of *S.* Enteritidis in the caeca, whereas systemic infection was occasionally seen from 48 hours on. Cell suspensions were analyzed by FACS. The macrophage function was tested *in vitro* by determining the nitric oxide production and the phagocytic and killing capacity of the cells. FACS analysis showed that the percentages of macrophages decreased in the spleen, but increased in the caeca during infection. Splenic macrophages seemed to increase their nitric oxide production during infection, whereas nitric oxide production in intestinal macrophages seemed unchanged. *In vitro* phagocytosis and killing of bacteria was increased in previously infected chickens in contrast to control chickens with a maximum at 48 hours post-infection. This result indicates that previous infection with *S.* Enteritidis increases the macrophage function in spleen and intestinal wall, due to either higher cell numbers or increased activity.

## INTRODUCTION

Poultry meat is the most popular animal food product worldwide, due to its nutritional, sensory and economical characteristics. However, poultry meat can be a source of zoonoses like *Salmonella*. *Salmonella* is a gram-negative, facultative intracellular pathogen, which is capable of infecting a variety of hosts. An infection with *Salmonella* usually starts via the oral route followed by colonization of the gut. Next, *Salmonella* is able to penetrate the epithelial barrier of the intestinal mucosa (Popiel and Turnbull, 1985). After phagocytosis of *Salmonella* by intestinal macrophages, present in the lamina propria, most if not all bacteria are killed by the intracellular killing mechanisms (Bloom and Boedeker, 1996). Because *Salmonella* is sometimes able to survive intracellularly in the macrophages, a systemic infection may occur by the dissemination of the bacteria to the spleen and liver (Bloom and Boedeker, 1996; Popiel and Turnbull, 1985). In addition, a specific immune response starts after phagocytosis of *Salmonella* when the macrophages process the bacteria and present the antigens on their surface and thereby stimulate the cellular and humoral immune responses. Hence, macrophages play an important role to clear the infection the first days post-infection (PI), but also later on in infection. In mammals, Maskell et al. showed that the initial suppression of bacterial growth in a primary *Salmonella* infection is a local event, probably mediated by the cellular response of the macrophages. So far, little is known about the early defense mechanisms occurring after *S.* Enteritidis infection in chickens, with respect to intestinal and splenic macrophages during the first days PI. In this study we investigated the numbers of macrophages in the gut and spleen and their functioning early after infection.

## MATERIAL AND METHODS

*Chickens*

Twenty 4-week-old White Leghorn chickens were housed in isolator facilities on wire bottom-cages. Chickens were provided with food and water ad libitum and they were observed daily. The birds were determined to be free of *Salmonella*. All chickens were cared for in accordance with accepted procedures of the Dutch law of animal welfare.

*Infection with S. Enteritidis*

After removing 5 animals from the isolator for necropsy as control animals, the other 15 animals were orally inoculated with 1 ml $1x10^9$ cfu *Salmonella* Enteritidis phage type 4 (nalidixic acid (Nal) resistant). *S.* Enteritidis was grown in Buffered Peptone Water (BPW) overnight with shaking at 150 rpm. Five chickens were sacrificed at 0 (control

animals), 24, 48 and 72 hours PI, and ileum, caecum, liver and spleen were removed aseptically and kept on ice. Caecum, liver and spleen were used for bacteriological examination and ileum, caecum and spleen were used for immunological research. Furthermore, small pieces of ileum and caecum were snap-frozen in liquid nitrogen, and stored at –20°C for immunocytochemistry.

*Bacteriological examination*

Bacteriological examination was performed according to routine procedures using BGA-Nal$^+$ plates.

*Macrophage isolation and functional assays*

The preparation of the spleen, ileum and caecum cell suspensions from the chickens were performed as been described previously for mice (Heijden and Stok, 1987). The phagocytic and killing capacity of intestinal and spleen macrophages was determined *in vitro* using $5x10^6$ spleen cells or $25x10^6$ ileum or caecum cells. Cells were incubated for 45 minutes 37°C with 50 µl (spleen) or 250 µl (ileum and caecum) *S.* Enteritidis (Nal resistant, $1x10^9$ cfu/ml, MOI =10). To kill extracellular bacteria 200 µg/ml gentamicin was added (45 minutes at 37°C). Cells were centrifuged and resolved in 1 ml RPMI with 5% FCS and 20 µg/ml gentamicin. After phagocytosis (directly after the first incubation with gentamicin) or after killing (12, 24, 48, 72 and 96 hours after *in vitro* killing) 1 ml 1% saponin in PBS was added to lyse the cells (5 minutes, room temperature). The number of *S.* Enteritidis internalized by the cells was determined by colony counting on BGA-Nal$^+$ plates. Prior to *in vitro* infection, cells were also plated onto BGA-Nal$^+$ to confirm that the colonies detected in the *in vitro* infection did not result from the *in vivo* infection. The different cultures of *Salmonella* used each assay for *in vitro* infection were examined to confirm that the suspensions contained approximately the same number of bacteria. Nitric oxide production by the macrophages was measured after 24 hours of incubation ($25x10^6$ cells, 37°C) with *E.* Coli LPS (LPS antigen, 10 µg/ml) or specific *S.* Enteritidis antigen, SeAg3, 1 µg/ml (Kramer et al., 1999).

*Flowcytometry and immunohistochemistry*

Using standard flowcytometry, the percentages of leukocytes (using MAb HISC7 specific for CD45 (Jeurissen et al., 1988a)), T-cells (using MAb anti-CD3 (Southern Biotechnology Associates, Birmingham, AL 35226)), B-cells (using MAb HISC1 specific for Bu1 (Jeurissen et al., 1988a)) and macrophages (with CVI-ChNL-68.1 (Jeurissen et al., 1988b)) were determined in ileum, caecum and spleen. Cryostat sections of the caeca and ileum were air-dried, fixed and stained using MAb as has been described previously (Jeurissen et al., 1988b).

*Statistical analysis*

The data were analyzed by general analysis of variance (ANOVA) with the statistical program Genstat (Genstat 5, Release 4.1, Fourth edition). The model of analysis tested the effects of the different days PI.

## RESULTS

*Bacteriological examination*

In control chickens, *S.* Enteritidis could not be detected in the various organs. All caeca of infected chickens were colonized with *S.* Enteritidis (Table 3.1). In the caeca, the number of *Salmonella* gradually increased significantly ($P = 0.02$) following the days PI. During the infection, all infected birds were systemically infected in liver and spleen with *S.* Enteritidis, although the infection level was very low and often only detectable after enrichment.

**Table 3.1.** Results of *Salmonella* colonization in the organs from chickens inoculated with *S.* Enteritidis.

| | Caecum | | Liver | | Spleen | |
|---|---|---|---|---|---|---|
| | levels | i.a.e.[A] | incidence[B] | i.a.e.[A] | incidence[B] | i.a.e.[A] |
| Control | 0 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 |
| 24 hours pi | $>10^4$ | 5/5 | 0/5 | 5/5 | 2/5 | 4/5 |
| 48 hours pi | $2.2 \times 10^8$ | 5/5 | 2/5 | 4/5 | 0/5 | 3/5 |
| 72 hours pi | $1.9 \times 10^9$ | 5/5 | 0/5 | 3/5 | 5/5 | 5/5 |

[A] i.e.a. = Incidence (No. infected / total per group) after enrichment

[B] No. infected / total per group

Macrophage function

The phagocytic and killing activity of splenic macrophages is depicted in Figure 3.1. It is shown that the phagocytic function of the control animals is lower compared to the previously infected animals. Moreover, the chickens that were previously infected by *S.* Enteritidis *in vivo* were able to kill the intracellular bacteria after *in vitro* infection faster. *Salmonella* multiplied within the cells from the control animals for 48 hours post *in vitro* infection. In contrast, *in vivo* infected chickens showed an increase in the number of intracellular *S.* Enteritidis during 12 hours after *in vitro* infection but then they were able to kill the bacteria. Chickens sacrificed 24 hours PI needed more time to start killing the intracellular *S.* Enteritidis compared to birds that were *in vivo* infected 48 or 72 hours

previously. Especially the chickens, that were infected 48 hours previously, showed a rapid decrease in the number of intracellular *S.* Enteritidis within 12 hours post *in vitro* infection.

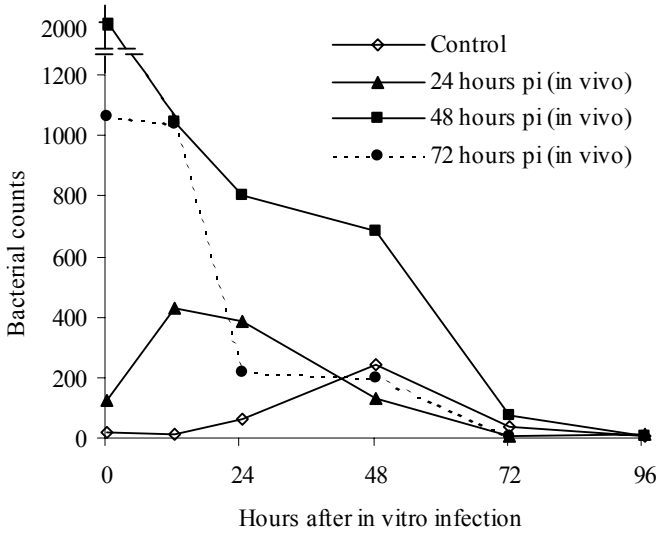


**Figure 3.1.** Phagocytic and killing activity of *S.* Enteritidis within spleen cells. Significant differences in days after *in vivo* infection were found 0, 12, 24 and 48 hours after *in vitro* infection ($P < 0.01$).

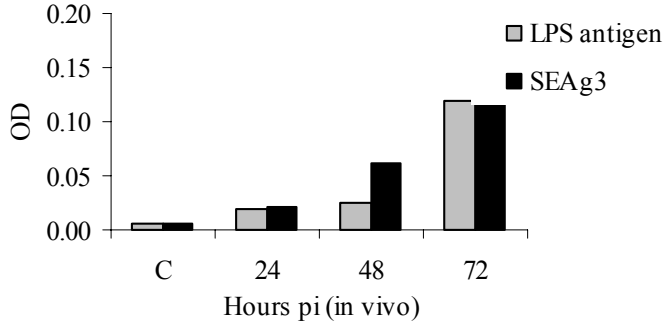


**Figure 3.2.** Nitric oxide production after stimulation with a nonspecific antigen (*E.* Coli LPS antigen) or with a specific *S.* Enteritidis antigen (SEAg3). The OD pictured is the OD of stimulated cells minus the OD of non-stimulated cells. Significant differences were found between the different hours pi for the spleen on both antigens ($P < 0.001$).

Nitric oxide production by spleen macrophages after *in vitro* stimulation with both antigens increased significantly after *in vivo* infection (Figure 3.2). Production of nitric oxide by the intestinal macrophages after *in vitro* stimulation was hardly detectable or reproducible at any time point after *in vivo* infection (results not shown).

*FACS analysis and immunocytochemistry*

The percentage of T-cells in the spleen remained approximately the same during the experiment. In contrast, the percentage of B-cells and macrophages decreased slightly (Figure 3.3). The percentage of leukocytes in the intestine (both ileum and caecum) increased significantly during *in vivo* infection (until 48 hours PI), which was also shown in the percentage of macrophages and T-cells. At necropsy at 48 and 72 hours PI, the intestine showed symptoms of inflammation. Upon light microscopic examination, it was shown that *S.* Enteritidis invaded the mucosa of the ileum and caecum and caused edema and an influx of leukocytes (results not shown).

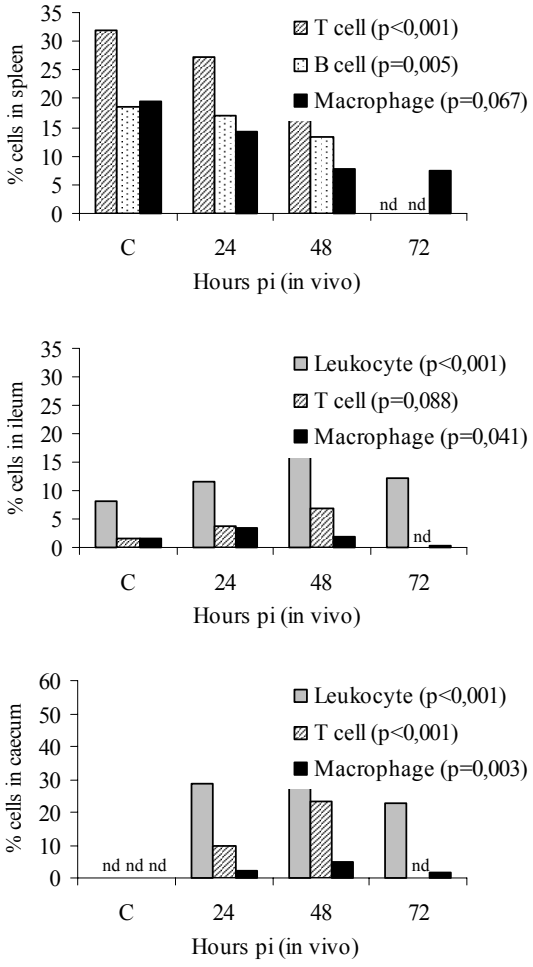


**Figure 3.3.** FACS analysis of living cells isolated from different organs (nd = not done). Significant differences in hours pi for the various cell types in the different organs were depicted behind the legend.

## DISCUSSION

In this experiment, the number and the function of splenic and intestinal macrophages early after *in vivo* infection with *S.* Enteritidis was investigated in chickens. The results confirm that previous *in vivo* infection with *S.* Enteritidis increased the activity of the splenic macrophages *in vitro*. This conclusion was drawn from the results that during *in vivo* infection the phagocytic and killing activity, as well as the nitric oxide production of splenic macrophages was increased (Figure 3.1 and 3.2). Furthermore, the numbers of leukocytes in the intestinal mucosa increased during infection whereas the percentage of macrophages in the spleen decreased according FACS analysis (Figure 3.3). An infection with *S.* Enteritidis thus influences the distribution of various immune cells in spleen and gut probably indicating a migration of leukocytes from the spleen to the infected intestinal tissue. Within the population of leukocytes the percentages of splenic macrophages decreased during infection, this in contrast to the percentages T and B lymphocytes in the spleen that remained stable the first 72 hours PI. In ileum and caecum, the percentages of leukocytes, T lymphocytes and macrophages increased the first 48 hours PI of infection. Previous studies also showed no significant differences in T ($CD4^+$ and $CD8^+$) and B ($IgG^+$) lymphocyte subpopulations in the spleen early after infection, only the percentages of splenic IgA+ or IgM+ B lymphocytes increased significantly after oral *S.* Enteritidis infection (Sasai et al., 1997). An influx of heterophils and macrophages in the lamina propria early after infection with *S.* Enteritidis has also been shown previously (Desmidt, 1999).

Infection with *S.* Enteritidis results in a higher activity of the macrophages to clear the infection. Although the percentage of the macrophages decreased in the spleen during infection, we found that the phagocytic and killing activity of nonopsonized *S.* Enteritidis and the nitric oxide production after stimulation increased significantly. Phagocytic activity of chicken blood macrophages / monocytes is supposed to be even more effective after opsonization of the bacteria before *in vitro* phagocytosis, because then almost 95% of the bacteria are killed within 120 minutes after opsonization compared to 15% by the nonopsonized bacteria (Stabler et al., 1994). During a primary infection of *Salmonella*, however, specific antibodies are not present to facilitate phagocytosis and killing by the inflammatory cells, therefore we examined these parameters without opsonization. Even then, the function of the macrophages was increased in both spleen and intestine. Our results on the production of nitric oxide by intestinal and spleen macrophages showed a large individual variation between chickens. In addition birds with a higher percentage of macrophages did not automatically showed a higher nitric oxide production. This variation might be due to the genetic background of the chickens, small differences in the isolation

procedure or possibly heterophils in the suspension. Desmidt studied the plasma levels of nitric oxide in chickens infected with *S.* Enteritidis showing that the levels increased after *in vivo* infection. They furthermore showed that the increase in nitric oxide was dependent on the dose of *S.* Enteritidis (Desmidt, 1999). In conclusion, our study suggests that the number and functioning of the macrophages from spleen and intestinal wall is increased after a previous infection with *S.* Enteritidis. Further research will focus on the question whether the resident macrophages in the intestinal wall of chickens and their functioning correlate with the development of carrier animals with respect to *Salmonella*.

## ACKNOWLEDGEMENT

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# Chapter 4

## COMPARISON OF NATURAL RESISTANCE IN SEVEN GENETIC GROUPS OF MEAT-TYPE CHICKEN

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**ABSTRACT**

Several studies have shown that genetic variation exists in response to various *Salmonella* strains in mammals and poultry. In the current study immunocompetence traits related to natural resistance to *Salmonella* were measured in seven genetic groups of meat type chickens (in total 296 chickens involved). Therefore, parameters were measured of both innate (phagocytic activity) and adaptive immune responses that are important after a natural or experimental *S.* Enteritidis infection. Two traditional Old Dutch Breeds, (groups 1 and 2), four commercial broiler groups (groups 3 to 6), and one experimental broiler group (group 7) were used. In two periods, birds of each group were sacrificed at ages between 14 to 35 days post hatch. Significant differences between groups were found for most immune parameters measured. Moreover, significant correlations were found between various immune parameters. Results suggest that each group (each chicken) gave rise to an immune response, using the innate or the adaptive immune response. Overall in the current study, group 2 showed the highest natural resistance. None of the groups though was uniformly superior with respect to all traits measured. To conclude, for reliable measurements of general immunocompetence or resistance to *e.g. Salmonella*, it is important to measure several parts of the immune system.

## INTRODUCTION

Over the last 40 years, meat-type chickens have mainly been selected for rapid growth, percentage breast meat and improved feed efficiency. Combined with changes in feed and housing, this has led to a more than doubling of growth rate. Poultry meat or poultry products can be a source of zoonoses like *Salmonella* Enteritidis. There are indications that chickens and turkeys that have been selected for meat production have become more susceptible to various infections (Nestor et al., 1996; Li et al., 1999; Rao et al., 1999; Li et al. 2000; Yunis et al., 2000) and routine use of antibiotics became standard. Recently the use of preventive and non-therapeutic antibiotics and vaccines to prevent disease or infections in animals has come under criticism. In order to maintain the present health status, natural disease resistance is thus becoming more important. Natural disease resistance has been described as the inherent capacity of an animal to resist disease when exposed to pathogens, without prior exposure to immunization (Hutt, 1958). Both innate and adaptive immune responses are important aspects of natural disease resistance.

Differences in innate and adaptive immune responses between various chicken lines are influenced by genetic differences in natural disease resistance (James et al., 1983; Kramer et al., 2001). Various studies already described the differences in susceptibility between various chickens lines in response to *S.* Enteritidis (Bumstead and Barrow, 1988; 1993; Guillot et al., 1995; Protais et al., 1996; Duchet Suchaux et al., 1997; Girard Santosuosso et al., 1998; Kramer et al., 2001). The innate immune response is considered to be the first line of immunological defense to infective agents like *Salmonella*. Phagocytes (*e.g.*, monocytes, macrophages, and heterophils) represent an important component of the innate immune response, because they are able to internalize *Salmonella* for destruction, however *Salmonella* is also able to survive within these phagocytes (Stabler et al., 1994). In addition, these cells start the adaptive immune response by presentation of antigenic fragments of *Salmonella* to the T- and B-cells (Dietert et al., 1991). After recognition, T-cells become stimulated and are able to eliminate *Salmonella* or stimulate B-cells. The stimulated B-cells will produce antibodies in intestinal secretion like bile or blood to clear the infection (Barrow, 1992; Desmidt et al., 1996).

To the authors knowledge, no research has been reported to establish whether natural resistance to *S.* Enteritidis differs on a fundamental physiological level between genetically different groups of chickens. Therefore, the objective of the current study was to demonstrate the differences in natural resistance between genetic groups by measuring differences in immunocompetence between seven different meat-type chicken groups. Therefore, parameters were measured of both innate and adaptive immune responses that are important after a natural or experimental *S.* Enteritidis infection and the correlations between these parameters were calculated.

## MATERIAL AND METHODS

*Animals*

Seven genetically different meat-type chicken groups were used for this experiment (Table 4.1). They included two traditional Old Dutch Breeds, group 1 (Barnevelder) and 2 (Noord Hollandse Blauwe), obtained from IPC dier, Barneveld, The Netherlands. These Old Dutch Breeds have a high mature body weight, although the weight of these chickens is much lower compared to modern mature broilers. Furthermore four modern outbred broiler groups were included, group 3 (meat-type), group 4 (meat-type but also selected for reproduction), group 5 (offspring of group 3 × group 4 cross), and group 6 (commercial cross of Ross breeders. Groups 3, 4, and 5 were kindly provided by Hybro B.V. (Boxmeer, The Netherlands) and group 6 was obtained from Praktijkonderzoek Beekbergen (Beekbergen, the Netherlands). Finally, group 7 was included which is an experimental broiler selected for improved feed conversion (obtained from ID-Lelystad, The Netherlands). After hatch, it was determined that birds were free of *Salmonella*. In total, 296 uniquely identified birds were used (both males and females), as shown in Table 4.1. Each group was housed separately, and was divided over different cages (two to four cages per group and sexes were mixed). Chickens were fed a ration containing 20.9% CP and 13.1 MJ ME/kg. Birds were given feed and water ad libitum and were observed daily. All chickens were cared for in accordance with accepted procedures of the Dutch law of animal welfare. The animal experiment committee from the institute approved all experimental procedures applied to the birds.

**Table 4.1.** Number of chickens sacrificed per group per period.

| Genetic group | Period | | Total | Sex | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | | Male | Female | Unknown |
| 1 | 23 | 21 | 44 | 19 | 22 | 3 |
| 2 | 7 | 10 | 17 | 9 | 6 | 2 |
| 3 | 33 | 25 | 58 | 28 | 25 | 5 |
| 4 | 31 | 21 | 52 | 20 | 29 | 3 |
| 5 | 0 | 39 | 39 | 11 | 27 | 1 |
| 6 | 20 | 16 | 36 | 23 | 12 | 1 |
| 7 | 30 | 20 | 50 | 22 | 24 | 4 |
| Total | 144 | 152 | 296 | 132 | 145 | 19 |

*Necropsies*

Necropsies started from the age of 2 weeks. The experiment was started at this age because the level of maternal antibodies that could be present in the Old Dutch Breeds (parents of these birds could not be investigated for the presence of any pathogens) will be vanished by that time. Parents of the broiler groups were determined to be free of *Salmonella* or other specific pathogens, thus no maternal antibodies will be present in the offspring from groups 3 to 7. Not more than 16 to 20 animals could be included in the necropsy per day, because of the number of immunological parameters that were measured. The experiment was divided over two periods (Table 4.1) of 3 weeks with a 1-week interval for logistic reasons. Overall, the number of chickens that were sacrificed per day was equally divided over the period as much as possible (at least 2 animals per group per day were used, more if available). For each day of necropsy, the birds were selected randomly from the cages, both males and females. From group 2, only 17 chickens were available in total due to poor hatching results, so animals could not be sacrificed each day of necropsy. Furthermore, group 5 was only available in the second period (this cross could not be provided in the first period), but these chicks were derived from the same sources. At necropsy, before killing, total body weight of each chicken was measured and blood samples were taken, then chickens were anaesthetized and killed using $CO_2$. Ileum, caecum, and spleen were removed aseptically and kept on ice until immunological examination.

*Leukocyte isolation from the intestine and spleen*

Isolation of leukocytes from the ileum, caecum, and spleen was performed as described previously (Kramer et al., 2001). In short, both ileum and caecum was isolated, opened longitudinally, rinsed thoroughly with PBS and cut in 0.5 to 1 cm pieces. All tissue pieces were incubated for 10 to 15 minutes in PBS containing 0.145 mg/ml dithiotreithol and 0.37 mg/ml EDTA in a shaking water bath (110 strokes/minute, 37˚C). The pieces of ileum and caecum were rinsed once with RPMI 1640 (GibcoBRL, Lifetechnologies Ltd, Paisley, Scotland) containing 5% fetal calf serum (FCS) and incubated in RPMI 1640 supplemented with 5% FCS, 0.15 mg/ml collagenase, and 0.1 mg/ml DNase in a shaking water bath (200 strokes/minute, 37˚C) during 75 to 90 minutes. The supernatant and the pieces of ileum and caecum were squeezed through a 70 µm nylon gauze (Cell strainer Falcon 2350, Becton Dickinson, Leiden, The Netherlands) to obtain a single cell suspension. For leukocyte isolation from the spleen, the spleen was cut in small pieces and incubated in RPMI 1640 with 1 mg/ml collagenase for 10 minutes at 37˚C and squeezed through a 70 µm nylon gauze. This cell suspension was centrifuged, re-suspended in 20 ml Dulbecco's Modified Eagle Medium (DMEM) and loaded on a ficoll gradient (12 ml) to isolate the mononuclear cells from the spleen, performed according to routine procedures. These single cell suspensions from ileum, caecum and spleen were used to measure the entry and survival

of *S.* Enteritidis. In addition, the nitric oxide production and the unstimulated ex-vivo lymphocyte proliferation assay of the splenic leukocytes were measured.

*Nitric oxide production*

Nitric oxide (NO) production by the splenic leukocytes was measured with and without *in vitro* stimulation, using *Escherichia* Coli lipopolysaccharide antigen (LPS). In this assay, 50 µl single cell suspension ($2.5 \times 10^6$ cells) was mixed with 50 µl RPMI1640 plus 10% FCS with or without LPS (10 µg/ml) and incubated for 28 hours (37˚C, 5% $CO_2$). Then, 50 µl of the supernatant was mixed with 50 µl Griess-reagents, and the absorption was measured using the Spectramax 340 (Molecular Devices, Sunnyvale, CA, 94089) at 550 nm. Then the NO concentration (µM) was calculated by interpolation from a standard curve.

*Entry and survival assay*

The entry and survival of nalidixic acid resistant *S.* Enteritidis PT4 in leukocytes present in cell suspensions isolated from spleen, liver and caecum was performed as described previously, with a few changes (Kramer et al., 2001; 2003). Single cell suspensions of ileum, caecum, and spleen (1 ml containing $5 \times 10^6$ cells) were incubated in triple with 100 µl of a fresh overnight culture of *S.* Enteritidis ($\pm 5 \times 10^8$ cfu/ml, MOI = 10) for 30 minutes at 37˚C. Then, 200 µg/ml gentamicin was added to kill extracellular bacteria (45 minutes, 37˚C). To measure the number of bacteria that entered the cells, one of the three identical suspensions was centrifuged and 1 ml 1% saponin in PBS was added to lyse the cells during 5 minutes at room temperature. The number of *S.* Enteritidis internalized by the cells was examined on Brilliant Green Agar plates enriched with nalidixic acid. To measure the survival of *S.* Enteritidis, the two other cell suspensions were washed, 1 ml RPMI1640 plus 5% FCS and 20 µg/ml gentamicin was added and the cells were further incubated for another 14 hours or 24 hours at 37˚C, 5% $CO_2$. Then the cells were lysed using saponin and the number of living bacteria was examined using the same procedure as described earlier.

*Unstimulated ex-vivo Lymphocyte Proliferation Assay*

The T-cell activity was measured using the unstimulated lymphocyte proliferation assay (LPA). Per well of a 96-wells cell culture cluster plate (Costar, Corning, Schiphol-Rijk, The Netherlands), $1 \times 10^6$ cells of the single cell suspension of the spleen was incubated in 150 µl RPMI1640 enriched with 1% normal chicken serum (41˚C, 5% $CO_2$). After 68 hours of incubation, 0.5 µCi 3H-thymidine was added per well. After a further 4 hours of incubation, the cells were harvested and the amount of incorporated tritium counted on a beta-counter (1450 Microbeta plus, EG&G Wallac, Breda, The Netherlands).

*Total Ig concentrations*

The B-cell activity was measured using total (natural) IgM, IgG, and IgA concentrations. The antibody concentrations were measured in sera collected from all animals in the experiment using a double antibody sandwich-ELISA (DAS-ELISA). Between all incubation steps, the plates were washed using water added with 1% Tween 80 before further incubation. For the DAS-ELISA, 96 wells high binding capacity plates (Greiner, Alphen a/d Rijn, The Netherlands) were coated with 100 µl monoclonal antibody (Mab) anti IgG (CVI-ChIgG-47.3), anti IgM (CVI-ChIgM-59.7) or anti IgA (CVI-ChIgA-46.5) in carbonate buffer (pH 9.6) overnight at 37˚C and thereafter used immediately. All Mab were purchased from ID-Lelystad (Lelystad, The Netherlands), the specificity of these Mab was described previously (Bianci et al., 1990). Then the plates were incubated with 150 µl ELISA buffer (0.5 M NaCl in 0.01 M phosphate buffer, pH 7.2) with 0.05% Tween 80 and 1% milkpowder (Skim Milk, Becton Dickinson, Sparks, MD, 21152) per well for 30 minutes at 37°C. After that, the sera were added in serial twofold dilutions (predilution 1:1000) in 100 µl ELISA buffer with 0.05% Tween 80 and 0.25% milkpowder and the plates were incubated at 37°C for 1 hours. Next, 100 µl ELISA buffer containing biotin-labelled anti light chain (CVI-ChIgL-47.5, ID-Lelystad) with 0.05% Tween 80 and 0.25% milkpowder was added and incubated for 1 hour at 37˚C. Then peroxidase conjugated avidine (DAKO A/S, Denmark) was added and incubated for 1 hour at 37°C. Finally, the substrate solution (0.005% $H_2O_2$ and 1 mg/ml tetramethylbenzidine in 0.1 M sodium-acetate and 0.1 M citric acid buffer, pH 6.0) was added and after 5 to 7 minutes incubation at room temperature, the reaction was stopped by adding 50 µl 0.5 M $H_2SO_4$. The absorption was measured at 450 nm using the Spectramax 340. The slope of each curve was determined, using this slope and the known concentration of the control samples on each plate the concentrations of all samples were calculated.

*Data analysis*

Due to the unbalanced design of this experiment, data were analyzed using Restricted Maximum Likelihood (REML) analysis (Genstat 5, Release 4.2, Fifth edition, VSN International Ltd, Oxford, UK). A model with the factors group, periods and sex and a covariate for age was used for the analysis:

$$Y_{ijk} = \mu + group_i + age + period_j + sex_k + group_i \times age_i + e_{ijk}$$

Where µ = overall mean, group = fixed effect for the i-th group, age is a covariate for age, period is a fixed effect for the j-th period, sex is a fixed effect for the k-th sex and e = residual. Initially, the effect of the different cages per group was also taken into account as a random effect. However, as these cage effects were not significant, it was not included

in the final model. Prior to analysis, all variables except body weight and antibody concentrations were transformed to natural logarithmic scale to normalize variances. The results shown in the tables and in the figures are the predicted means that are adjusted to the mean of the covariate age. The standard errors were calculated for the predicted means. As all parameters, except the antibody concentrations and body weight, were transformed, the standard errors of the transformed parameters could not be presented after backtransformation. Therefore, their transformed means together with their standard errors, and the means after backtransformation, which are more informative, are presented. Of the entry and survival assay only the transformed data are given (because of the large number of data). All significant effects and interactions are given below each table or figure. If there was a significant interaction of group × age, the groups responsible for this interaction were identified, because only the predicted means of group were presented to enhance the clarity of the results. The approximate Least Significant Difference (LSD) at $P < 0.05$ was calculated using predicted means and standard errors of the differences (SED), two groups differed significantly when the difference between the means of two groups was higher than 1.96 × SED between those groups. The predicted means, together with the LSD were used to evaluate the data. The residuals from the REML analyses, being deviations of the various variables from a common adopted model, were used to calculate the correlations between the variables.

**Table 4.2.** Nitric oxide production of stimulated and unstimulated spleen cells

| Genetic group | Nitric oxide production, μ*M* | | | | | |
|---|---|---|---|---|---|---|
| | Without stimulation[1] | | | With LPS stimulation[2] | | |
| | LN[3,4] (means) | ± SEM | Mean (after back transformation) | LN[3,5] (means) | ± SEM | Mean (after back transformation) |
| 1 | 2.4 | 0.1 | 10.5 | 2.9 | 0.1 | 17.8 |
| 2 | 2.3 | 0.2 | 9.7 | 3.1 | 0.2 | 21.3 |
| 3 | 2.1 | 0.1 | 8.2 | 2.8 | 0.1 | 16.6 |
| 4 | 1.8 | 0.1 | 6.2 | 2.5 | 0.1 | 12.2 |
| 5 | 2.0 | 0.2 | 7.0 | 2.6 | 0.1 | 13.9 |
| 6 | 2.1 | 0.2 | 8.0 | 2.8 | 0.1 | 16.4 |
| 7 | 1.9 | 0.1 | 6.9 | 2.6 | 0.1 | 13.5 |

[1] Significant effects were group ($P = 0.04$), age ($P < 0.001$), period ($P = 0.003$).
[2] Significant effects were age ($P = 0.002$).
[3] LN = Natural Logarithmic
[4] Least Significant Difference = 0.3
[5] Least Significant Difference = 0.3

## RESULTS

*Innate immune responses*

Nitric oxide production by splenic leukocytes was measured after stimulation by *E.* Coli LPS and without stimulation (Table 4.2). Group differences were found for the NO production by unstimulated cells ($P = 0.039$). Groups 1 and 2 gave the highest responses without LPS stimulation and also the highest responses with LPS stimulation ($P = 0.093$). Moreover, groups 4 and 5 showed the lowest NO production both with and without stimulation with LPS.

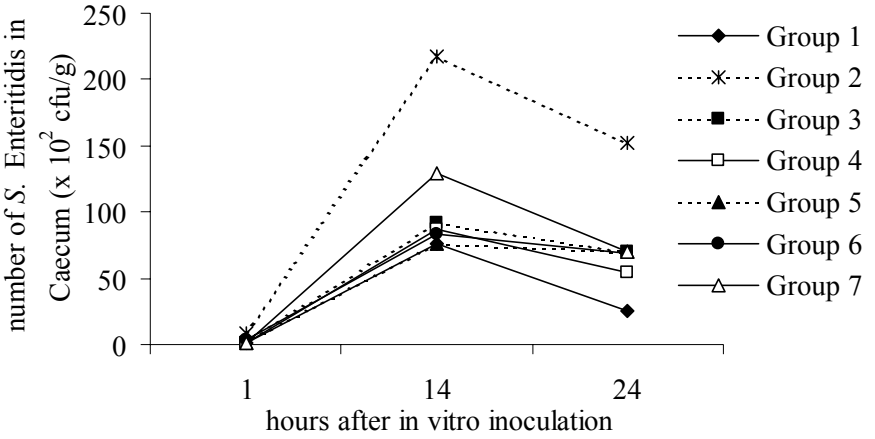
The results on entry and survival of *S.* Enteritidis in leukocytes isolated from the caecum, ileum and spleen are presented in Figure 4.1. The number of *S.* Enteritidis that have entered the cells was very low for both caecum and ileum leukocytes, so not many differences were found. However, in the ileum, caecum and splenic leukocytes, group 2 showed the highest number of bacteria that had entered the cells. Overall, the number of bacteria that had entered the splenic leukocytes was higher than the number of bacteria that entered the ileum and caecum leukocytes.

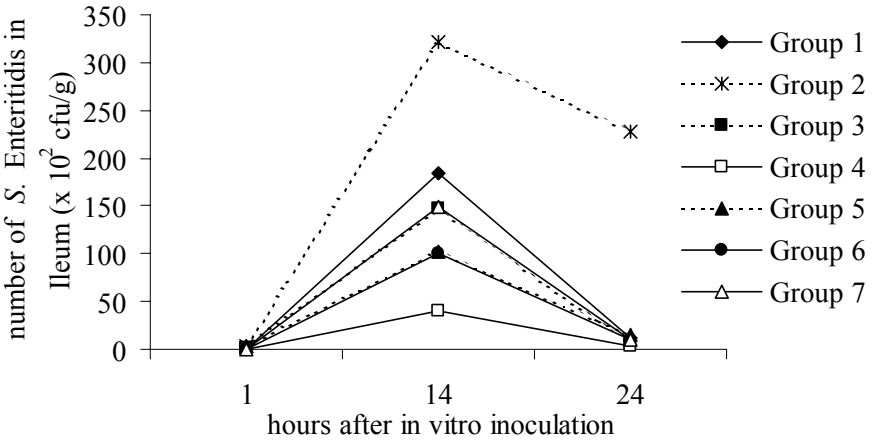
Survival of *S.* Enteritidis in the leukocytes was measured 14 hours and 24 hours post *in vitro* inoculation (Figure 4.1). Differences between groups were found in the caecum and the spleen, 14 and 24 hours post-inoculation. In all three organs (caecum, ileum, and spleen), the number of intracellular living bacteria increased between 0 and 14 hours post-inoculation and decreased between 14 and 24 hours post-inoculation. Most remarkable are the results of group 2. A lot of *S.* Enteritidis survived in cells of both caecum and ileum of group 2 after 14 and 24 hours of incubation, because a high number of viable *S.* Enteritidis were present in the cells. This is in contrast to splenic cells of group 2, showing the lowest number of *S.* Enteritidis in the cells of all groups at both 14 and 24 hours post-inoculation. Furthermore, chickens of the other Old Dutch Breed, group 1, also showed low survival of *S.* Enteritidis in the splenic leukocytes.

*Adaptive immune responses*

Results of the transformed and backtransformed counts of the T-cell responses (LPA) are shown in Table 4.3. A significant difference was found for group, age and period ($P < 0.001$). Overall, results suggest that group 3 and 5 have a higher lymphocyte proliferation than the other groups, whereas the Dutch Breeds groups 1 and 2 take an intermediate position in LPA.

With the assay used to measure B-cell responses, all natural antibody concentrations were measured produced against all kind of unknown and undefined antigens


number of S. Enteritidis in Caecum (x 10² cfu/g)
250
200
150
100
50
0
1
14
24
hours after in vitro inoculation
Group 1
Group 2
Group 3
Group 4
Group 5
Group 6
Group 7


number of S. Enteritidis in Ileum (x 10² cfu/g)
350
300
250
200
150
100
50
0
1
14
24
hours after in vitro inoculation
Group 1
Group 2
Group 3
Group 4
Group 5
Group 6
Group 7

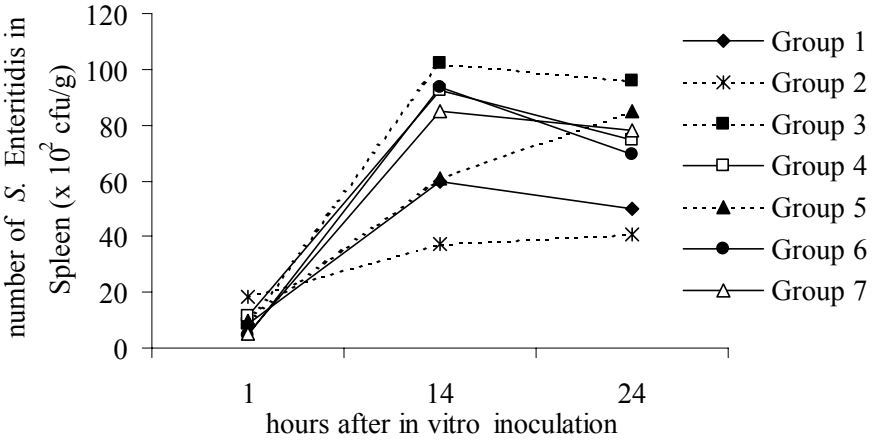

number of S. Enteritidis in Spleen (x 10² cfu/g)
120
100
80
60
40
20
0
1
14
24
hours after in vitro inoculation
Group 1
Group 2
Group 3
Group 4
Group 5
Group 6
Group 7

**Figure 4.1.** Entry and survival (at time = 14 hours and time = 24 hours) of *S.* Enteritidis in leukocytes from various organs of each genetic group after an *in vitro* inoculation of *S.* Enteritidis. The number of *S.* Enteritidis that entered in all cells of each organ was determined by counting colonies that grew on BGA-Nal$^+$ plates. For the caecum, the effect age ($P < 0.001$) was significant for survival to 14 h, and group ($P < 0.001$), age ($P < 0.001$), and period ($P = 0.014$) was significant for survival to 24 hours. For the ileum, the effect age ($P < 0.001$) and period ($P < 0.001$) was significant for entry, period ($P = 0.001$) was significant for survival to 14 h, and period ($P = 0.012$) for survival to 24 hours. For the spleen, the effect age ($P = 0.031$) was significant for entry, group ($P = 0.009$), age ($P = 0.002$) and sex ($P = 0.025$) was significant for survival to 14 h, and group ($P < 0.001$), age ($P < 0.001$), and group × age ($P = 0.01$) for survival to 24 hours. Group 2 differed significantly from groups 1,3,4,5, and 6 at 14 hours after inoculation and from group 4 at 24 hours after inoculation in caecum cells. Further more, group 1 was significantly lower than all other groups at 24 hours after inoculation in caecum. In ileum cells, group 4 was significantly lower than groups 1, 2, 3, and 7 at 14 hours after inoculation, and group 2 was significantly higher than all other groups at 24 hours after inoculation. In spleen cells, group 2 was significantly lower than groups 3, 4, 6, and 7 at 14 hours after inoculation, and lower than group 3, 4, 5, and 7 at 24 hours after inoculation. Furthermore in spleen cells, group 1 was significantly higher than group 3 at 14 hours after inoculation, and higher than group 3, 4, 5, and 7 at 24 hours after inoculation and finally, group 3 was significantly higher than group 5 at 14 hours after inoculation.

the chicks had encountered in their lifetime. The total IgM, IgG and IgA antibody concentrations are presented in Figure 4.2. For IgM the interaction group × age ($P < 0.001$) was found to be significant. This was mainly caused by groups 1 and 2 that showed the highest concentrations at all ages compared to the other groups, moreover the IgM concentration of group 2 increased to a greater extent over time compared to the other groups (data not shown). However overall, there was a trend that IgM concentrations of all groups increased with time. Like for IgM, the interaction group × age ($P < 0.001$) for IgG was also found to be significant. This interaction was caused by the two extremes: group 1

**Table 4.3.** Unstimulated lymphocyte proliferation assay.

| Genetic group | Lymphocyte proliferation assay, counts[1] | | |
|---|---|---|---|
| | LN[2,3] (means) | ± SEM | Mean (after backtransformation) |
| 1 | 7.1 | ± 0.2 | 1176 |
| 2 | 7.2 | ± 0.4 | 1277 |
| 3 | 7.6 | ± 0.2 | 1910 |
| 4 | 7.1 | ± 0.2 | 1235 |
| 5 | 7.3 | ± 0.3 | 1431 |
| 6 | 6.9 | ± 0.3 | 1036 |
| 7 | 6.7 | ± 0.2 | 831 |

[1] The effects of group ($P = 0.006$), age ($P < 0.001$), and period ($P < 0.001$) were significant.
[2] LN = Natural Logarithmic
[3] Least Significant Difference = 0.6

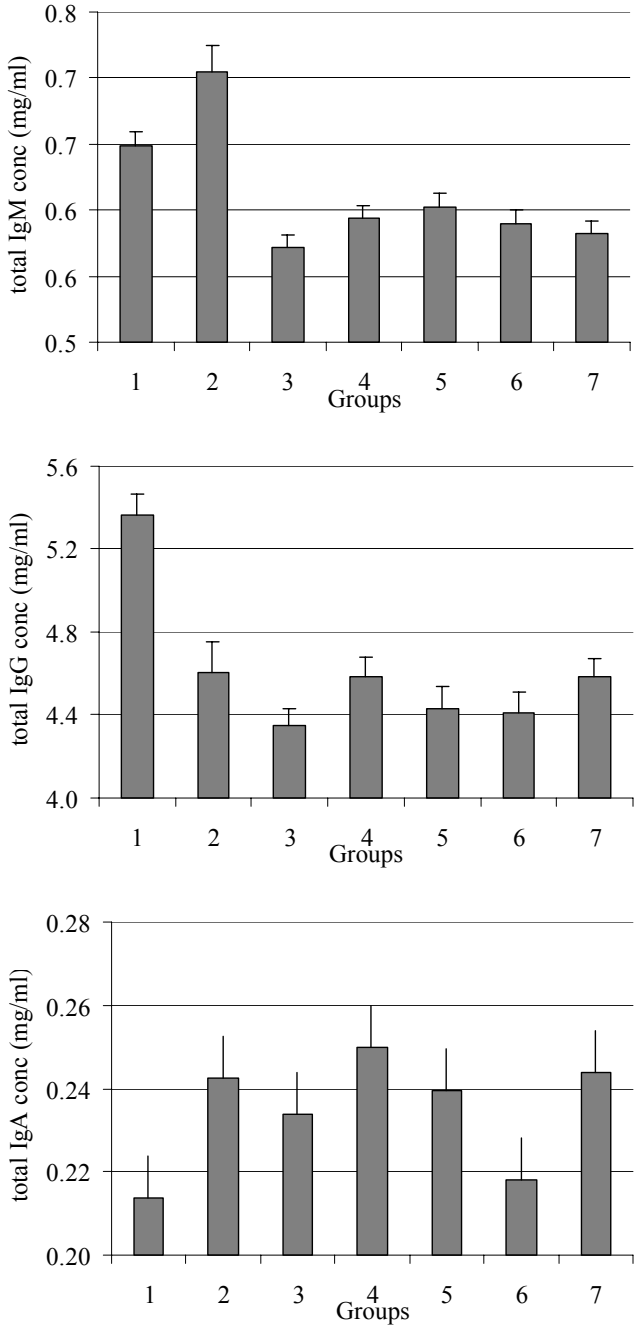



**Figure 4.2.** Total antibody concentration in serum of all genetic groups. For total IgM concentration, the effects of group ($P < 0.001$), age ($P < 0.001$), period ($P = 0.022$), sex ($P = 0.013$) and group × age ($P < 0.001$) were significant. For total IgG concentration the effects of group ($P < 0.001$), age ($P < 0.001$), sex ($P = 0.032$) and group × age ($P < 0.001$) were significant. For total IgA concentration effects of group ($P = 0.003$), age ($P < 0.001$) and sex ($P = 0.003$) were significant. Least Significant Difference (LSD) for IgM is 0.03, for IgG LSD = 0.24 and for IgA LSD = 0.02.

that showed the highest IgG concentrations and group 5 showed the lowest IgG concentration. The latter concentration increased by age, so that after 30 days its concentration was comparable to the other groups. Without these two groups (group 1 and 5) no significant effect of group was found in the IgG concentration. For the IgA concentrations, significant differences between groups were found ($P < 0.001$).

For both IgM and IgG, groups 1 and 2 showed the highest total antibody concentrations compared to groups 3 and 5 that showed the lowest antibody concentrations. Although group 1 showed high IgG and IgM concentrations, the IgA concentrations of this group was the lowest of all groups. In contrast, group 2 not only showed high IgG and IgM concentrations, but also its IgA concentrations were high compared to the other groups.

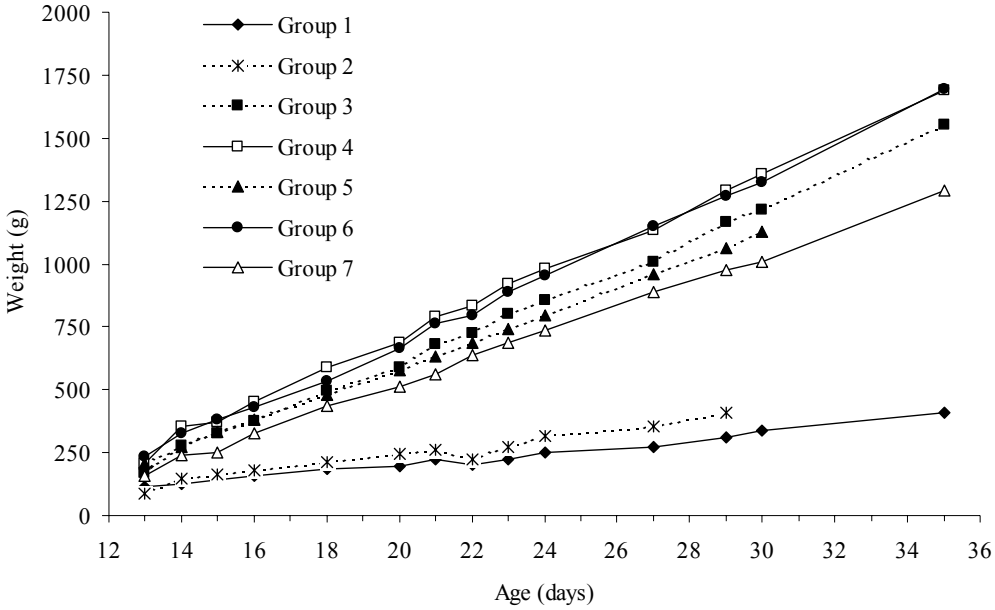



**Figure 4.3.** Body weight of chickens of the genetic groups during the experiment. The effects of group, age, sex and group × age ($P < 0.001$) were significant. From 15 days on, significant differences were found in body weight between the Old Dutch Breeds (group 1 and 2) and the broiler groups (group 3 to 7). From 24 days on, significant differences in body weight were also found between group 7 and group 4.

*Body weight*

Figure 4.3 shows the body weight of the different genetic groups from 13 to 35 days of age. Considerable differences in body weight at 35 days of age were present between the four commercial broiler groups 3, 4, 5, and 6 (1.7 to1.8 kg), the experimental broiler group 7 (1.25 kg) and the Dutch Breeds, groups 1 and 2 (0.3 to 0.4 kg). These differences

**Table 4.4.** Correlations[1] between the different tests, after correction for the fixed effects[2]

| | LPA[3] | NO (-LPS)[4] | NO (+LPS)[4] | Caecum entry | Caecum survival time = 14 h | Caecum survival time = 24 h | Ileum entry | Ileum survival time = 14 h | Ileum survival time = 24 h | Spleen entry | Spleen survival time = 14 h | Spleen survival time = 24 h | IgA | IgG | IgM |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LPA | 1.00 | 0.11 | 0.13 | 0.20 | 0.15 | 0.08 | 0.21 | 0.05 | 0.12 | 0.02 | –0.03 | –0.13 | 0.00 | –0.07 | 0.06 |
| NO (-LPS) | | 1.00 | 0.85 | –0.12 | –0.16 | –0.12 | –0.02 | –0.32 | –0.25 | –0.06 | 0.12 | 0.33 | –0.04 | 0.01 | 0.02 |
| NO (+LPS) | | | 1.00 | –0.10 | –0.11 | –0.13 | 0.00 | –0.30 | –0.18 | –0.02 | 0.18 | 0.35 | –0.04 | 0.03 | 0.03 |
| Caecum entry | | | | 1.00 | 0.33 | 0.18 | 0.35 | 0.30 | 0.27 | 0.21 | –0.08 | –0.15 | –0.14 | –0.20 | –0.03 |
| Caecum survival time = 14 h | | | | | 1.00 | 0.57 | 0.28 | 0.15 | 0.28 | 0.08 | 0.20 | -0.02 | –0.07 | –0.06 | –0.01 |
| Caecum survival time = 24 h | | | | | | 1.00 | 0.13 | 0.12 | 0.23 | 0.06 | –0.01 | –0.02 | –0.04 | 0.03 | 0.10 |
| Ileum entry | | | | | | | 1.00 | 0.22 | 0.16 | 0.12 | 0.01 | –0.09 | –0.02 | –0.05 | 0.09 |
| Ileum survival time = 14 h | | | | | | | | 1.00 | 0.63 | 0.24 | –0.03 | –0.15 | –0.07 | –0.17 | –0.07 |
| Ileum survival time = 24 h | | | | | | | | | 1.00 | 0.18 | 0.07 | –0.05 | –0.04 | –0.16 | –0.10 |
| Spleen entry | | | | | | | | | | 1.00 | 0.01 | 0.04 | –0.08 | –0.23 | –0.03 |
| Spleen survival time = 14 h | | | | | | | | | | | 1.00 | 0.58 | –0.24 | –0.01 | –0.16 |
| Spleen survival time = 24 h | | | | | | | | | | | | 1.00 | –0.08 | 0.01 | –0.05 |
| IgA | | | | | | | | | | | | | 1.00 | 0.13 | 0.30 |
| IgG | | | | | | | | | | | | | | 1.00 | 0.42 |
| IgM | | | | | | | | | | | | | | | 1.00 |

[1] $|r| > 0.11$ ($P < 0.05$) and $|r| > 0.16$ ($P < 0.01$),
[2] fixed effects are group, age, period and sex
[3] LPA = lymphocyte proliferation assay
[4] NO(-LPS) = nitric oxide (NO) production of unstimulated spleen cells, NO(+LPS) = nitric oxide production of spleen cells stimulated with lipopolysaccharide (LPS)

reflect the effect of selection in commercial broiler groups over the last 50 years on high body weight within 6 weeks compared to the Old Dutch Breeds. A significant interaction was present for group × age ($P < 0.001$) and a significant effect was found for group ($P < 0.001$). The significant interaction reflects the fact that body weights are diverging over time (Figure 4.3).

*Correlations between the tests*

Many residual correlations found between the tests were significant, although most correlations were not very strong ($|r| < 0.5$, Table 4.4). The entry and survival of *S.* Enteritidis in the cells isolated from the caecum showed highly significant positive correlations with the entry and survival of *S.* Enteritidis in the cells isolated from the ileum. Furthermore, the entrance of *S.* Enteritidis into the splenic cells also showed a significant positive correlation with the number of *S.* Enteritidis that entered the cells of both caecum and ileum. The survival of *S.* Enteritidis in both caecum and ileum leukocytes demonstrated a significant negative correlation with the NO production in both unstimulated and stimulated cells. This is in contrast with the significant positive correlation of the survival of *S.* Enteritidis in the spleen cells with the NO production. Correlations among the different antibody concentrations (IgG, IgM, and IgA) were significant. Although not all correlations were significant, almost all correlations between the entry and survival of *S.* Enteritidis in the caecum, ileum, and splenic cells on the one hand and the antibody concentrations on the other hand were negative. Furthermore, a positive correlation was found between the unstimulated LPA and the NO assay.

## DISCUSSION

In the present study the differences in baseline profiles of various aspects of natural resistance important in resistance to *S.* Enteritidis were described in seven genetic groups of meat-type chicken (four commercial groups, one experimental broiler group and two Dutch Breed groups). The natural resistance was determined by measuring both innate and adaptive immune parameters.

Two assays (NO production and entry and survival of *S.* Enteritidis) were used to measure part of the innate immune responses, and the adaptive immune responses were determined by measuring the total (natural) antibody concentrations and the unstimulated lymphocyte proliferation. After chickens become infected with *S.* Enteritidis, *S.* Enteritidis will enter several cell types of the immune system. After entrance of the bacteria, these cells will start to produce *e.g.* cytokines or radicals like NO to respond to the infection. The entry and survival of *S.* Enteritidis in various cell suspensions derived from different organs (ileum, caecum and spleen) was measured. Some immune cell types (*e.g.* phagocytes) are

able to kill a lot of *S.* Enteritidis that entered the cell within a few minutes, however, most cell types (*e.g.* macrophages, T-cells, and B-cells) are not able to fully clear all bacteria within 48 hours (Kramer, 2002). Although it is shown that *Salmonella* is able to survive within various cell types (including lymphocytes) for a longer period, phagocytes are the main population of cells that can actively internalize *Salmonella*. To measure the different parameters, both the numbers of *S.* Enteritidis that had entered the various leukocytes within 30 minutes were measured, and the survival of *S.* Enteritidis at 14 and 24 hours post-inoculation was determined separately. The NO production was measured in splenic cell suspensions, which contained various cell types. Even though NO is produced by all sorts of cells like certain T-and B-cells, natural killer cells and mast cells, NO is mainly produced by phagocytes after stimulation with an antigen, like *Salmonella*. Although it is not entirely correct to mention NO production by LPS stimulated cells a parameter for natural resistance, the data collected showed a highly significant correlation ($r = 0.85$, $P < 0.001$) between the NO production of the stimulated and unstimulated cells. In other words, both assays showed similar results as to the relative activity of the cells.

Broiler groups 3 to 7, selected for a high body weight, showed a lower natural resistance in at least one part of the innate or adaptive immune response compared to the Old Dutch Breeds, group 1 and 2. Groups 1 and 2 showed higher NO production by the splenic leukocytes, the lowest survival of *S.* Enteritidis in the splenic leukocytes and a higher total IgM and IgG antibody concentration compared to the commercial broiler groups. Moreover, group 2 showed the highest entrance of *S.* Enteritidis in all three organs compared to the other groups. Qureshi and Havenstein (1994) showed that genetic selection for broilers towards enhanced performance traits negatively influenced the humoral immune response, moreover they found little or no effect on macrophage and natural killer cell functions. Similar negative relationships between selection for body weight and humoral immunocompetence have been reported (Martin et al., 1990; Miller et al., 1992; Pinard-van der Laan et al., 1998; Rao et al., 1999; Yunis et al., 2000). In other studies by Li and co-workers a lower mitogenic response to Concanavalin A of the peripheral blood mononuclear cells and higher antibody response to sheep red blood cells was found in turkeys that were selected for increased 16 weeks body weight (Li et al., 1999; 2000). The present results and the previous studies suggest that selection for high body weight in poultry can affect other physiological parameters like antibody concentrations or mitogenic responses as a part of natural resistance.

Results from the current study suggested that higher survival of *Salmonella* in the splenic leukocytes correlate with a higher NO production by the splenic leukocytes and a lower antibody concentration. In contrast to the spleen, lower survival of *Salmonella* in ileum and caecum leukocytes seemed to correlate with a higher NO production by splenic leukocytes, and like for splenic leukocytes, a lower antibody concentration. Moreover,

negative correlations were found between the survival of *Salmonella* in ileum leukocytes and splenic leukocytes. These results suggest that the entry and survival of *Salmonella* in ileum and caecum leukocytes might be controlled by parameters other than those associated with splenic leukocytes. It seems that after a chicken becomes infected with *Salmonella*, a systemic infection of *Salmonella* of the spleen can be cleared by a low survival of *Salmonella*, a low NO production by the splenic leukocytes and a high humoral response. Previously it was also observed that after *S.* Enteritidis infection of young chickens, a lower activity of phagocytes was correlated with a higher humoral and cellular activity, that seemed to prevent a systemic bacterial infection (Kramer et al., 2001). Although animals described in the present study were not experimentally infected *in vivo*, the same trend in immune response was found. This indicates that infection with *Salmonella* does not interfere with the pattern of natural resistance, but only induces higher levels of the immune response. Cheng and Lamont (1988) also found a significant negative correlation between entrance and T-cell mediated response of chickens after vaccination. Furthermore, Qureshi et al. (1986) and Qureshi and Miller (1991) studied several commercial broiler lines for different macrophage functions. They demonstrated that different broiler lines exhibit differences in their macrophage functional potential. Moreover they showed that the line that showed a reduced phagocytic activity and a early killing activity of the phagocytes for antigens also showed a reduced humoral response to different antigens (Qureshi and Miller, 1991).

We tend to conclude from these studies and the results of the current study that after effectively eradicating a pathogen like *S.* Enteritidis by the innate immune response (high activity of the phagocytes), the adaptive immune response is not highly stimulated (low T and B-cell activity). This negative relationship can be explained because the defensive functions of phagocytes come into effect immediately upon the invasion by pathogens, whereas the T- and B-cells need time to be stimulated before they respond to the pathogen. Thus, an individual with a strong activity of the phagocytes is able to fight the pathogen quickly, not allowing antigenic stimulation to persist for the time needed for the T- and B-cells to become stimulated. However, this does not mean that the T- and B-cell responses from this individual are defective.

Generally, in the current study group 2 showed the highest natural resistance compared to other groups. However, each group can rely on sufficiently responding by activation of a part of the immune system, the innate (phagocytes) or adaptive (T- and B-cells) immune response, for protection. Moreover, several significant correlations were found between the different immunological traits measured. However, none of the seven groups was uniformly superior for all immunological traits measured. In swine, differences were found in various immune assays measured and various positive and negative correlations were found between different immune assays, and there was also no group of animals uniformly superior for all immune assays measured (Edfors-Lilja et al., 1994).

In conclusion, the present study demonstrated differences and relationships among immunocompetence traits in natural resistance of chickens, with indications that this is related to resistance to a specific pathogen *S.* Enteritidis. The results suggest that each chicken group is able to express a proper immune response using predominantly either the innate or adaptive immune response after an infection. Based on the standard errors, sufficient variation is still present in these immunological traits of all groups to change the total immunocompetence or genetic resistance of the animals by selection. However, the current study and others (Qureshi and Miller, 1991; Qureshi and Havenstein, 1994; Boa-Amponsem et al., 1999) suggest that both for selective breeding and for adaptation to particular production conditions, it is important to include several parts of the immune system, since one arm of the immune system does not provide a reliable indication of general immunocompetence or resistance in general.

## ACKNOWLEDGMENTS

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# Chapter 5

## CHARACTERIZATION OF THE INNATE AND ADAPTIVE IMMUNITY TO *SALMONELLA* ENTERITIDIS PT4 INFECTION IN FOUR BROILER LINES

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**ABSTRACT**

Four broiler lines were inoculated orally with *Salmonella* Enteritidis phage type 1 at the age of 7 days (experiment A: line 1 and 2) and at the age of 1 day (experiment B: line 3 and 4). At various days post-infection chickens were sacrificed and the number of *Salmonella* in the caeca, liver and spleen were determined. Furthermore, phagocytic activity, cellular immune responses, and humoral responses were determined using, respectively, single-cell suspensions of spleen or intestine and serum. In both experiments, similar trends were seen. Increased numbers of *S.* Enteritidis were found in the caeca of lines 1 and 3, whereas at the same time a decreased colonization was found in the spleen and in the liver, as compared to lines 2 and 4. In the latter two lines, the phagocytic activity of the phagocytes was higher and the humoral responses were lower. Observations from this study suggest that lower activity of phagocytes and higher humoral activity prevent systemic *S.* Enteritidis infection.

## INTRODUCTION

Salmonellosis is a worldwide problem as a human disease and as an economical problem for poultry farmers. In fact, salmonellosis is one of the most common food-borne diseases in humans. Over the last decade, the use of antibiotics and attenuated vaccines to restrain or prevent *Salmonella* infection in domestic animals have been criticized because of the possible development of antibiotic resistant *Salmonella* and the potential dangers of residual antibiotics and vaccines in animal-derived food products for human consumption.

*Salmonella* is a facultative, intracellular pathogen which is capable of infecting a variety of hosts, sometimes resulting in disease. An infection with *Salmonella* usually starts via the oral route followed by colonization of the gut. In mice and humans, *Salmonella* has been shown to penetrate the mucosal epithelium of the small intestine using M cells after attachment to the intestinal epithelium (Neutra and Kraehenbuhl, 1992). The interaction between *Salmonella* and the epithelium triggers the chemotaxis of phagocytic cells to the site of infection (Desmidt et al., 1997; Henderson et al., 1999) and the phagocytic cells start to eliminate the bacterial pathogen. Nevertheless, *Salmonella* is able to survive and replicate within macrophages (Buchmeier and Heffron, 1989; Abshire and Neidhardt, 1993; Stabler et al., 1994). Hence, macrophages play a role in the dissemination of *Salmonella* from the gut to various organs, such as liver and spleen (Carter and Collins, 1974; Popiel and Turnbull, 1985).

A number of studies have indicated that considerable genetic variation exists in response to viral (Bumstead, 1998), bacterial (Adams and Templeton, 1998), and parasitic (Stear and Wakelin, 1998) pathogens between different chicken lines. Hutt (1958) described natural disease resistance as the inherent capacity of an animal to resist disease when exposed to pathogens, without prior exposure to immunization. The earliest examples of differences in susceptibility of chickens to *Salmonella* infection are described in the 1940s and 1950s (Hutt and Scholes, 1941; Smith, 1956). More recently, Bumstead and Barrow (1988, 1993) showed large differences in susceptibility to different serotypes of *Salmonella* (*S.* typhimurium, *S.* gallinarum, *S.* pullorum and *S.* Enteritidis) between inbred lines of White Leghorn chickens. These chicken lines were either resistant or susceptible to all the different serotypes of *Salmonella*, suggesting that there may be a general mechanism of resistance to different serotypes of *Salmonella* in chickens. In France, four different chicken lines were investigated for variability in the resistance to *Salmonella* Enteritidis phage type 4. One experimental broiler line was used, one inbred Leghorn type and two outbred Leghorn chickens. These different lines showed differences in mortality rates or caecal colonization (Protais et al., 1996; Duchet Suchaux et al., 1997; Girard Santosuosso et al., 1998). The results of the different research groups are difficult to compare, because part of

the observed variation in *Salmonella* resistance is related to age, infection dose and route, environmental factors (*e.g.* stress), and food intake (Bumstead and Barrow, 1988; Nakamura et al., 1994a; Nisbet et al., 1994; Klasing, 1998). Nevertheless, a significant component of variation in disease resistance and susceptibility to *Salmonella* infection appears to be heritable and therefore to be stably passed from parent to offspring (Bumstaed and Barrow, 1988, 1993). However, the mechanism behind disease resistance has been investigated to a limited extent only in the previous studies.

The aim of this study is to identify the differences in the innate and adaptive immune response after an oral infection with *S.* Enteritidis phage type 4 in four different outbred broiler lines. Particularly, the phagocytes (monocytes, macrophages and heterophils) might play an important role in the dissemination of *Salmonella*, since these cells are the main component of the innate immune response after an infection and *Salmonella* is able to survive within the macrophages (Stabler et al., 1994). Because chicks are very sensitive for an infection, the first days post-hatch, the differences in susceptibility were compared between an infection at the age of 1 day and 1 week in two separate experiments. We determined the number of *Salmonella* in the caeca, liver, and the spleen and the phagocytic activity of the phagocytes, the cellular immune response and the humoral immune response.

**Table 5.1.** The number of chicks for both experiments sacrificed on the different days post-infection.

| Experiment | Line | | Days post-infection (DPI) | | | | | Dead[a] | Total[b] |
|---|---|---|---|---|---|---|---|---|---|
| | | | 0 | 3 | 7 | 14 | 28 | | |
| Exp A | 1 | Control | 3 | 2 | 2 | 1 | 2 | 2 | 12 |
| | | Infected | | 4 | 4 | 4 | 4 | | 16 |
| | 2 | Control | 4 | 2 | 2 | 2 | 2 | | 12 |
| | | Infected | | 4 | 4 | 4 | 3 | 1 | 16 |
| Exp B | 3 | Control | | | 5 | 5 | 3 | 2 (2) [c] | 17 |
| | | Infected | | | 15 | 15 | 19 | 8 | 57 |
| | 4 | Control | | | 5 | 5 | 5 | 0 | 15 |
| | | Infected | | | 15 | 15 | 21 | 5 | 56 |

[a]Birds that died during the experiment.

[b]Total 201 birds were used for the experiments.

[c]Before the experiment started two chicks died (between parenthesis) and during the experiment two control birds died.

## MATERIALS AND METHODS

*Chickens*

In experiment A (Exp A), two different outbred broiler lines from ID-Lelystad were tested: line 1 (selected for high body weight) and line 2 (selected for low feed conversion). In total, 28 male broilers were included of both lines 1 and 2 (Table 5.1). Within each line, half sibling progeny of four sires were selected. In experiment B (Exp B), two outbred broiler lines (kindly provided by Euribrid, Boxmeer) were used: line 3 (meat-type, a nucleus sire line) and line 4 (meat-type and also selected for reproduction, a nucleus dam line). In total, 145 male broilers have been tested, 74 chicks of line 3 and 71 chicks of line 4 (Table 5.1). This full sibling progeny originates from 15 sires and dams within each line. All flocks were determined to be free of *Salmonella*.

*Housing*

After hatching, all chicks were kept in isolator facilities on wire bottom cages. In each experiment, control animals (that were not infected) of both lines were housed together. The infected chicks were kept separate from the control animals and each line was housed separately. Moreover, in Exp B both lines 3 and 4 were divided over two separate isolators. All isolation facilities received the same airflow. Animals were provided with food and water ad libitum and were observed daily. All chickens were cared for in accordance with accepted procedures of the Dutch law of animal welfare.

*Infection with S. Enteritidis*

*S.* Enteritidis phage type 4 (nalidixic acid (Nal) resistant) was grown in buffered peptone water (BPW) overnight with shaking at 150 rpm. In Exp A, 7-day-old chicks were orally inoculated with 0.25 ml of the bacterial challenge suspension with $3.6 \times 10^5$ *S.* Enteritidis CFU. Chicks of Exp B were inoculated at the age of 1 day with 0.25 ml containing $8.5 \times 10^5$ *S.* Enteritidis CFU.

*Necropsies*

The number of chicks that were sacrificed at the different days post-infection (DPI) in both experiments is shown in Table 1. In both experiments, one infected animal for each of the sires was used in both experiments at each DPI and one control animal for each of the sires was sacrificed during the experiments. Since the number of chicken in Exp B was too high too deal with on 1 day, they were equally divided over 2 days (1 day before and 1 day after DPI). For both experiments, blood was collected to prepare serum to determine specific humoral immune responses. Then chickens were killed and ileum, caecum, liver, and spleen

were removed aseptically and kept on ice. Caecum, liver, and spleen were used for bacteriological examination and the ileum and spleen were used for immunological research.

*Bacteriological examination*

A 1 g of caecal content of each broiler was homogenized in 9 ml BPW, serial diluted in BPW, and plated onto brilliant green agar with nalidixic acid (BGA-Nal$^+$) for quantitative *Salmonella* determination. These BGA plates and the $10^{-1}$ dilution of the homogenate were incubated at 37ºC. When at the lowest dilution no bacteria were found, the $10^{-1}$ dilution was plated on BGA-Nal$^+$. Similarly, 1 g of liver or spleen tissue of each broiler was ground separately in 9 ml BPW in a Colworth Stomacher 400 (A.J. Steward company Ltd., London, UK) for 30 s, followed by selective plating with serial dilutions on BGA-Nal$^+$, as described above.

*Phagocyte isolation from gut and spleen*

Isolation of phagocytes was performed as described previously for plasma cells (Heijden and Stok, 1987). Briefly, the intestine (ileum) was isolated, opened longitudinally, rinsed thoroughly with PBS and cut in 0.5-1 cm pieces. All tissue pieces were incubated for 10-15 minutes in PBS containing 0.145 mg/ml dithiotreithol (DTT) and 0.37 mg/ml EDTA in a shaking water bath (110 strokes/minute, 37ºC). The pieces of small intestine were rinsed once with RPMI 1640 containing 5% FCS and 20 mM HEPES and incubated in RPMI 1640 supplemented with 5% FCS, 20 mM HEPES, 0.15 mg/ml collagenase, and 0.1 mg/ml DNase in a shaking water bath (200 strokes/minute, 37ºC) during 75-90 minutes. The supernatant and the pieces of intestine were subsequently squeezed through 250, 100, and 50 µm nylon gauze using RPMI 1640 containing 5% FCS, 20mM HEPES and 0.1 mg/ml DNase. In Exp B, an adjustment was made to this protocol. After the first two incubation steps, the supernatant and the pieces of intestine were squeezed only through a 70 µm nylon gauze (Cell strainer Falcon 2350, Becton Dickinson, Leiden, The Netherlands). In addition to Exp B, phagocytes were also isolated from the spleen. Therefore, the spleen was cut in small pieces, incubated in RPMI 1640 with 1 mg/ml collagenase for 10 minutes at 37ºC, and squeezed through a 70 µm nylon gauze. The resulting suspension was centrifuged, the cell pellet was re-suspended in 20 ml DMEM and loaded on a ficoll gradient (12 ml) to isolate the mononuclear cells, performed according to routine procedures.

*Phagocyte activity*

The phagocytic capacity of the phagocytes was determined. The phagocytic activity of the intestinal phagocytes in Exp A was measured using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT assay, Stevens and Olsen, 1993). $5 \times 10^6$ CFU of *S.* Enteritidis were added to 1 ml containing $2 \times 10^7$ ileum cells (1% macrophages, MOI=25)

and incubated for 90 minutes at 37ºC. The cells were lysed by adding 50 µl 0.2% saponin in PBS for 15 minutes. MTT (25 µl of 5 mg/ml in PBS) was added for 30 minutes to stain and precipitate the remaining living bacteria. The precipitation was dissolved by adding 100 µl of MTT buffer overnight (37ºC). The extinction coefficient was determined on the ELISA reader (Spectramax 340 Molecular devices, Sunnyvale, USA) at 595 nm.

In Exp B, the phagocytic activity of the spleen and gut cells was measured using colony counting on BGA-Nal$^+$ plates. Therefore, the cells were centrifuged, resolved and incubated with 1 ml of $1 \times 10^8$ CFU *S.* Enteritidis in RPMI for the gut $25 \times 10^6$ cells (1% macrophages and MOI=400) were used and for the spleen $5 \times 10^6$ cells (20% macrophages, MOI=100) and incubated for 30 minutes at 37ºC. To kill the extracellular bacteria, 200 µg/ml gentamycin was added for 45 minutes at 37ºC. The suspension was centrifuged and 1 ml 1% saponin in PBS was added to lyse the cells (5 minutes). The number of *S.* Enteritidis internalized by the cells was counted on BGA-Nal$^+$ plates.

In both experiments, a higher value indicated a higher phagocytic activity of the phagocytes.

*Lymphocyte Stimulation Test (LST)*

To obtain a single-cell suspension of the spleen, the same protocol was used as described previously for phagocyte isolation from the spleen in Exp B. In Exp A, the first incubation with collagenase was omitted and the spleen was squeezed through a 70 µm nylon gauze before incubation. Spleen cells ($1 \times 10^6$ cells per well) were stimulated in triplicate with 150 µl Con-A (10 µg/ml, ICN Pharmaceuticals Inc, Costa Mesa, USA) or 150 µl specific *S.* Enteritidis antigen (SeAg3, 1 µg/ml, prepared by culture of *S.* Enteritidis in BPW, concentrated by centrifugation, washed with PBS, and inactivated by sonification (four times 30 seconds on ice) in RPMI enriched with 1% normal chicken serum (GibcoBrl Life technologies, Ettenleur, The Netherlands) and cultured for 68 hours at 41ºC, 5% $CO_2$. Then 0.5 µCi methyl 3H-thymidine (Amersham Pharmacia Biotech, Buckinghamshire, UK) was added per well. After a further 4 hours of incubation, the cells were harvested and the amount of incorporated tritium was counted on a beta-counter (1450 microbeta™ plus, EG&G Wallac, Breda, the Netherlands). Lymphocyte stimulation is defined as the ratio between stimulated and non-stimulated samples i.e. the stimulation index (SI).

*Humoral response*

Anti-*Salmonella* titers were measured in sera collected from all animals in both experiments at the different DPI essentially as described previously (van Zijderveld et al., 1992). Both IgM and IgG titers were measured separately using an indirect ELISA based on *S.* Enteritidis LPS.

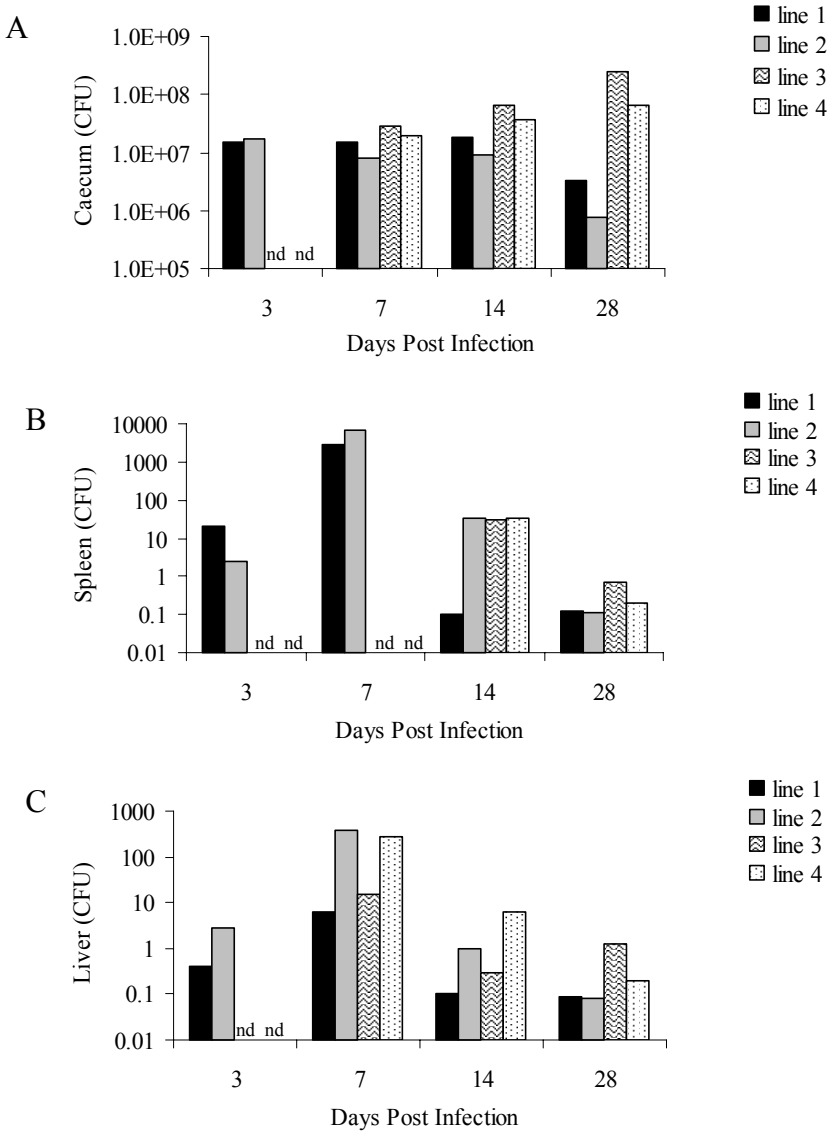


**Figure 5.1.** Results of *Salmonella* colonization in the different organs (caeca, liver, and spleen) from chickens inoculated with *S.* Enteritidis at the age of 7 days (experiment A, lines 1 and 2) or at the age of 1 day (experiment B, lines 3 and 4); nd: not determined. The significant differences and interactions found in the caecum were in Experiment B: DPI ($P < 0.001$) and line ($P < 0.05$), in the spleen in experiments A and B: DPI ($P < 0.001$), and in the liver in Experiments A and B: DPI ($P < 0.001$) and in Experiment B: line × DPI ($P < 0.001$).

*Flowcytometry*

Using standard flowcytometry, the percentages of leukocytes, T- and B-cells, and macrophages were determined in ileum and spleen. The cells were first indirectly stained with a MAb. The percentage of leukocytes was determined using a MAb HISC7 specific for CD45 (Jeurissen et al., 1988a), the T-cells using a MAb anti-CD3 (Southern Biotechnology Associates, Birmingham), the B-cells using a MAb HISC1 specific for Bu1 (Jeurissen et al., 1988a) and finally the macrophages with CVI-ChNL-68.1 (Jeurissen et al., 1988b). Next, fluorescein conjugated goat anti-mouse immunoglobulin isotype-specific antibodies (kindly provided by Dr. Sinkora from the Institute of Microbiology, Novy Hradek, Czech Republic) were used for the staining. Total 10,000 cells were analyzed using a FACSCalibur system (Becton Dickinson, Leiden, The Netherlands).

*Statistical analysis*

Data were analyzed using REML variance component analysis (Genstat 5, Release 4.1, fourth edition). Line, treatment (infected or control animals) and DPI were taken as fixed effects, whereas parents and individual animal number were taken as random affects. After REML analysis, residuals were used to study the correlation between various laboratory tests. The results shown are the predicted means of each group that were calculated after REML analysis. Some laboratory results (bacterial colonization in the caeca, liver, and spleen, and LST in both Exp A and Exp B and phagocytic activity in Exp B) were logarithmically (LN) transformed prior to analysis to improve the homogeneity of the variances for the different groups. The data were back transformed to the original scale and shown in the figures and tables.

## RESULTS

In Exp A, three birds died, two from the control group of line 1 and one from the infected group of line 2 with no detectable cause of death. In Exp B, four birds from the control group of line 3 died and 13 chickens from the infected group (eight of line 3 and five birds of the infected group of line 4 died). All but one died within 14 DPI with no detectable cause of death. In both experiments in the infected chickens that died, high numbers of *S.* Enteritidis were found in the caeca, spleen, and liver. However, time between death and necropsy is unknown, therefore no conclusions can be drawn from these data.

*S. Enteritidis colonization*

In the control animals, no *S.* Enteritidis could be detected in both experiments. For the infected animals, the results of the bacterial counts in the various organs are shown in Figure 5.1. All the caeca of the infected animals remained infected with *S.* Enteritidis in both

experiments. The infection levels of lines 1 and 2 remained stable during the first 14 DPI but appeared to decrease between 14 and 28 DPI. Differences found between line 1 and 2 were not significantly different, although it seemed that line 2 was able to eradicate *S.* Enteritidis more rapidly compared to line 1 (Figure 5.1A). In Exp B, line 3 carried significantly ($P < 0.05$) more *S.* Enteritidis compared to line 4 for each measurement. However, in contrast to Exp A, the numbers of *S.* Enteritidis in the caeca of both lines were still increasing up to 28 DPI.

The numbers of *S.* Enteritidis in liver and spleen were highest at 7 DPI in both experiments (Figure 5.1B and C), whereby all livers and spleens of Exp A were infected and most livers of Exp B. All the birds of Exp A cleared the infection 28 DPI, whereas in Exp B the infection sustained until 28 DPI in some birds. Overall in Exp A, line 2 was more severely systemically infected compared to line 1, which thus seemed able to clear the infection faster. Line 4 in Exp B was more severely systemically infected in the liver at 7 and 14 DPI compared to line 3. The number of livers and spleens that was still infected at 28 DPI was higher for line 4 compared to line 3. These results from both experiments showed that chickens from lines 2 and 4 remained more systemically infected during the experiment. This result seems to conflict the bacterial counts in the caeca that was lower in chickens from lines 2 and 4 and more severe in lines 1 and 3.

*Phagocyte activity*

The results of the phagocytic assay of Exp A (MTT assay) and Exp B (colony counts) are shown in, respectively, Tables 5.2 and 5.3. Due to the differences in number of bacteria between the overnight *Salmonella* cultures for the different DPI, only effects within each day can be compared. In Exp A, more *S.* Enteritidis were detected in the phagocytes of the control animals compared to the infected birds at 3, 7, and 14 DPI (Table 5.2). Thus *Salmonella* infection *in vivo* prior to the phagocytic assay reduced the survival of *S.* Enteritidis in the phagocytes. In addition, control and infected animals from line 2 showed a significantly higher phagocytic activity of *S.* Enteritidis at 3, 7, and 28 DPI compared to line 1.

**Table 5.2.** Phagocytic activity of gut cells of experiment A[a] (MTT incorporation).

| Line | Days post-infection (DPI) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 3 | | 7 | | 14 | | 28 | |
| | Control[b] | Infected[b] | Control | Infected | Control | Infected | Control | Infected |
| 1 | 0.281 | 0.091 | 0.072 | 0.057 | 0.874 | 0.571 | 0.086 | 0.117 |
| 2 | 0.632 | 0.387 | 0.426 | 0.209 | 0.657 | 0.572 | 0.107 | 0.136 |

[a]MTT incorporation; for line: $P < 0.001$; DPI: $P < 0.001$; treatment: $P < 0.01$; line × DPI: $P < 0.01$.

[b]The control animals were not infected by *S.* Enteritidis and the infected animals were orally inoculated with *S.* Enteritidis.

Obviously in Exp B, the phagocytic activity of the spleen cells was much higher compared to the gut cells (Table 5.3), because higher percentage phagocytic cells were present in the cell suspension from the spleen (analyzed by FACS analysis, Table 5.4). For the spleen of the infected animals, line 4 showed higher phagocytic activity compared to line 3 at 14 and 28 DPI. In contrast, for the gut of the infected animals, line 3 had higher phagocytic activity of *S.* Enteritidis. Like in Exp A, 28 DPI spleen cells of the infected chickens of Exp B also show lower phagocytic activity of *S.* Enteritidis in the phagocytes.

**Table 5.3.** Phagocytic activity of spleen and gut cells of birds from experiment B[a] .

| Tissue | Line | Days Post-Infection (DPI) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 7 | | 14 | | 28 | |
| | | Control | Infected | Control | Infected | Control | Infected |
| Spleen | 3 | 13 | 33 | 39 | 42 | 370 | 58 |
| | 4 | 27 | 32 | 28 | 48 | 277 | 83 |
| Gut | 3 | 1 | 2 | 7 | 8 | 10 | 25 |
| | 4 | 2 | 2 | 5 | 5 | 23 | 20 |

[a]Total number × $10^3$ of internalized bacteria by all cells by bacterial counting using BGA-Nal$^+$ plates; for the spleen DPI: $P < 0.001$; treatment × DPI: $P < 0.01$; the gut DPI: $P < 0.001$; treatment: $P < 0.05$.

*Cellular and humoral immune response*

In both experiments, spleen cells were stimulated with Con-A (data varied between SI of 3.5 and 89.7, results not shown) as a positive control, and with specific *S.* Enteritidis antigen (figure 5.2). The non-specific cellular immune response to Con-A was present at all ages in both experiments and no significant differences were found between lines and DPI within experiments. The specific response to *S.* Enteritidis antigen could be measured from

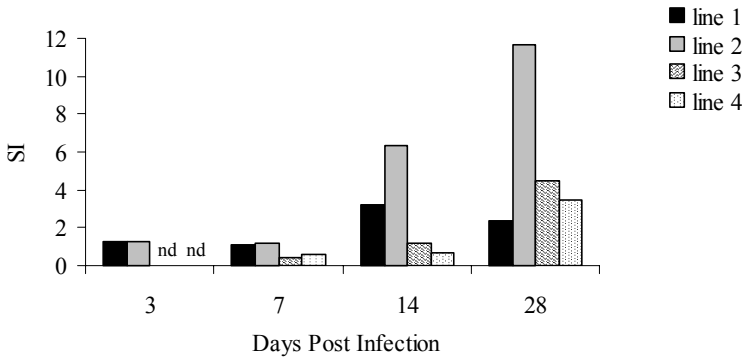


**Figure 5.2.** Results of the lymphocyte stimulation test (LST) after specific *Salmonella* antigen stimulation (SeAg3); nd: not determined. The significant differences and interactions found in the LST were in both Experiment A and B: DPI ($P < 0.001$), in experiment A: treatment × DPI ($P < 0.01$) and in Experiment B: line × DPI ($P < 0.001$), treatment ($P < 0.001$) and line ($P < 0.05$).

14 DPI onwards in Exp A, and only at 28 DPI in Exp B. In Exp A, line 2 showed a higher response compared to line 1, 14 and 28 DPI. In Exp B line 3 responded significantly ($P < 0.05$) higher compared to line 4, 28 DPI.

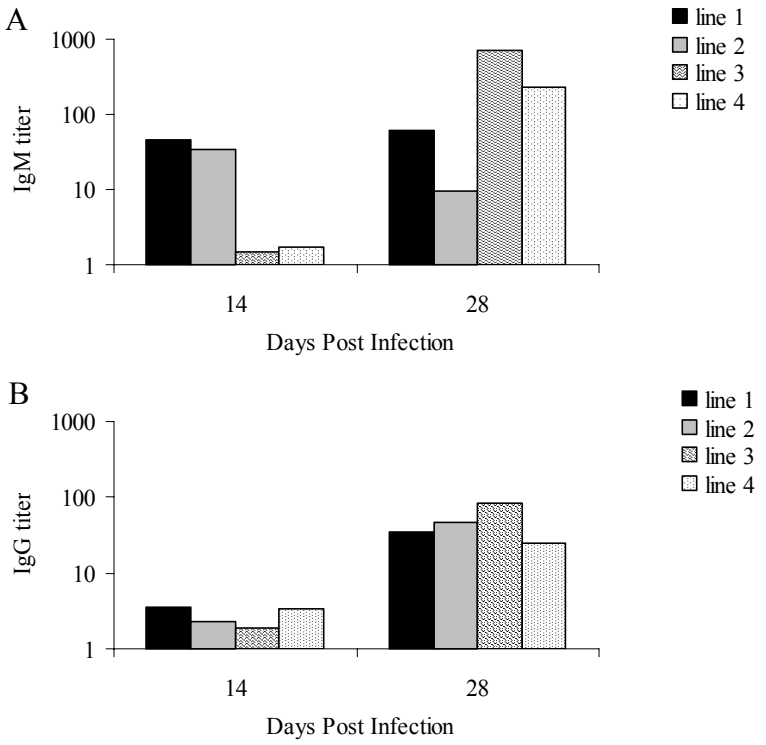


**Figure 5.3.** Humoral responses to *Salmonella enteritidis*, both IgM and IgG separately, of the infected birds from experiments A (lines 1 and 2) and B (lines 3 and 4). The significant differences and interactions found for IgM were in experiment A: line × treatment ($P < 0.001$) and treatment ($P < 0.025$) and in experiment B: treatment ($P < 0.05$) and line ($P < 0.01$) and for IgG in Experiment B: line ($P < 0.025$).

At 14 and 28 DPI, a specific IgM titer to *S.* Enteritidis could be detected (Figure 5.3A), whereas IgG could not be detected until 28 DPI in Exp A (Figure 5.3B). Line 1 showed a higher IgM response in the infected chicken compared to line 2. The interaction between line and treatment was highly significant ($P < 0.001$) for IgM. In contrast, line 2 tended to have a higher IgG response compared to line 1. In Exp B, both IgM and IgG were only detected at 28 DPI and line 3 showed a significantly ($P < 0.025$) higher humoral immune response (both IgM and IgG) than line 4 at 28 DPI.

*Flow cytometric analyses*

The cell suspension isolated from the gut and the spleen (the latter only in Exp B) was analyzed using FACS analysis (Table 5.4). In the gut, the percentage leukocytes varied from 3 to 15% of all cells, and macrophages varied from 0 to 4%. In the spleen, the percentages of the T- and B-cells increased with DPI; the T-cells increased from 32 to 42% and the B-cells from 15 to 27% in the infected chicks. The percentage of macrophages varied between 10 and 25% during the experiment. In Exp B, line 4 harbored more B-cells and significantly ($P < 0.001$) more macrophages in the spleen than line 3.

**Table 5.4.** FACS analysis from the spleen and ileum cell suspensions[a]

| Tissue | Cells | Line | Days Post-Infection (DPI) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 3 | | 7 | | 14 | | 28 | |
| | | | Control | Infected | Control | Infected | Control | Infected | Control | Infected |
| Spleen | T-cells[b] | 3 | | | 30.8 | 33.4 | 32.4 | 38.8 | 53.8 | 41.5 |
| | | 4 | | | 32.3 | 31.8 | 34.7 | 41.9 | 37.8 | 41.9 |
| | B-cells[c] | 3 | | | 20.9 | 15.0 | 23.4 | 22.0 | 24.1 | 26.6 |
| | | 4 | | | 19.9 | 16.1 | 26.9 | 26.8 | 26.1 | 27.3 |
| | Macrophages[d] | 3 | | | 11.7 | 18.1 | 13.8 | 10.7 | 16.1 | 17.4 |
| | | 4 | | | 12.9 | 26.2 | 19.9 | 16.8 | 17.7 | 20.4 |
| Gut | Leukocytes[e] | 1 | 13.1 | 9.2 | 5.4 | 7.1 | 3.3 | 5.6 | 8.4 | 6.0 |
| | | 2 | 6.1 | 6.5 | 3.0 | 5.4 | 2.9 | 5.2 | 6.4 | 5.3 |
| | | 3 | | | 11.7 | 10.9 | 10.1 | 9.2 | 15.7 | 15.5 |
| | | 4 | | | 11.7 | 10.0 | 11.2 | 12.7 | 13.6 | 14.7 |
| | Macrophages[f] | 1 | 4.9 | 1.4 | 4.2 | 2.3 | 4.2 | 3.0 | -3.0 | -0.3 |
| | | 2 | 0.1 | 0.3 | -1.4 | 5.5 | 1.4 | 4.1 | -1.0 | -0.4 |
| | | 3 | | | 2.6 | 1.0 | 0.5 | 1.0 | 4.2 | 2.7 |
| | | 4 | | | -0.8 | 1.5 | 0.8 | 1.8 | 2.2 | 4.1 |

[a] Percentage cells from total cell suspension.

[b] For experiment B, DPI: $P < 0.01$.

[c] For experiment B, DPI: $P < 0.01$; line × DPI: $P < 0.05$; treatment × DPI: $P < 0.01$.

[d] For experiment B, line: $P < 0.001$; DPI: $P < 0.001$; treatment: $P < 0.01$; treatment × DPI: $P < 0.001$.

[e] For experiment A, DPI: $P < 0.05$; for experiment B, DPI: $P < 0.001$.

[f] For experiment A, DPI: $P < 0.001$; treatment: $P < 0.025$; line × treatment: $P < 0.025$; line × treatment × DPI: $P < 0.025$; for Experiment B, DPI: $P < 0.01$.

*Sire and dam effects and relationships between the tests*

For each day of necropsy, one progeny of each pair of parents was sacrificed. In Exp B, differences were found between sires and dams within each line. Several effects of the parents approached significance, like for the phagocytic activity using spleen cells,

IgG response, and for most FACS analyses. Significant relationships between the tests in Exp B were for the phagocytic activity and the LST ($P < 0.001$) and for the IgM and IgG responses 28 DPI ($P < 0.01$).

## DISCUSSION

In these two experiments, *Salmonella* clearance was related to immunological responses of chickens, both cellular mediated (lymphocytes and phagocytic cells) and humoral (antibody) immune responses. Although, these two experiments with different lines differed in age of challenge, the results from both experiments suggest that an inverse relationship may exist between the severity of the caecal infection and the colonization in the systemic organs, liver and spleen (Figure 5.1). This inverse relationship may be explained by the phagocyte function (Tables 5.2 and 5.3) and the humoral responses (Figure 5.3). The relative high numbers of *S.* Enteritidis in the caeca and relative low numbers of *S.* Enteritidis in the systemic organs seems to be related to increased humoral responses and decreased phagocyte functions. Although, phagocytes were isolated from the spleen (Exp B) and ileum (Exp A and Exp B) and not the caecum, both ileum and caecum are part of the common mucosal immune system. Moreover, the colonization was not unambiguously related to the T-lymphocyte-dependent cellular immune response, because the results of both experiments were contrary to each other (Figure 5.2).

For disease resistance to *Salmonella*, a facultative intracellular bacterium, phagocytes (monocytes, macrophages and heterophils) represent an important component of the immune protection mechanism. Many factors are known that might affect the development immunological response to a pathogen, such as stress (Holt, 1992), diet (Knowles and Donaldson, 1998; Peterson et al., 1999) and age (Williams and Whittemore, 1975; Jeurissen et al., 1989; Holt et al., 1999). Mainly, the latter could have an enormous effect, because in young animals shortly after hatch, the immune system is immature and only partially developed. Because age seems to affect the severity of infection by *Salmonella* (and have the highest risk to be infected in the field situation), we chose to compare the birds infected at the age of 1 day (Exp B) or 7 days (Exp A).

*S.* Enteritidis colonization in both spleen and liver after an infection of 1-day-old chickens sustained longer compared to the chickens infected at the age of 7 days. Moreover, the number of bacteria in the caeca infected at 1 day of age was still increasing until 28 DPI, while the infection in chickens infected at 7 days of age was decreasing. Also the lymphocyte stimulation and the humoral responses in Exp B were not detectable until 28 DPI, compared to these responses in Exp A which were already present at 14 DPI. These results suggest that birds have more difficulties to clear an infection of *S.* Enteritidis when they are infected shortly after hatch. This result was also found by Desmidt et al. in 1997,

who compared two groups of White Leghorn chickens infected with *S.* Enteritidis at the age of 1 day or at 4 weeks of age. The 4-week-old chickens cleared the *S.* Enteritidis infection much faster. In conjunction, Duchet-Suchaux et al. (1995) found high mortality rates after an oral infection with *S.* Enteritidis at the age of 1 day, whereas these mortality rates were not found when White Leghorn chickens were infected at the age of 1 or 3 weeks. They also found that the organ colonization in the chickens infected at the age of 3 weeks was weak and transient. Our data show that 1-week-old chickens cleared the systemic infection 4 weeks post-infection and 1-day-old chickens cleared the infection even slower. Thus, results from our experiments and these studies clearly indicate that the age at which the chickens become infected is important for the ability of the chickens to clear systemic infection and thus for the horizontal transmission.

Differences between chicken lines in the clearance to *Salmonella* have been extensively studied (Lindell et al., 1994; Protais et al., 1996; Duchet Suchaux et al., 1997; Girard Santosuosso et al., 1998). Nevertheless, studies on the interaction between the immunological responses and the capability to clear *S.* Enteritidis infection between lines was not investigated in great detail, except for the humoral immune responses. Our results suggest that higher humoral responses are related to a lower systemic infection and a higher caecal carrier state and these results are comparable to those of others (Arnold and Holt, 1995; Desmidt et al., 1998). The elimination of *S.* Enteritidis partly depends on the humoral immunity, whereby the local response in the gut appeared more effective than the systemic response (Desmidt et al., 1998). After treatment with cyclophosphamide or testosterone propionate to suppress the humoral immune system and subsequent oral infection with *S.* Enteritidis, the intestinal shedding of *S.* Enteritidis was increased compared to untreated chickens, but the dissemination to the spleen was not different from controls (Arnold and Holt, 1995). Apart from the humoral response, we found differences between lines in phagocytic activity of nonopsonized *Salmonella*. The phagocytic activity from the spleen cells (Exp B) played an important role in the clearance of *Salmonella* from the spleen and thus the systemic infection. We found that lines (lines 2 and 4) with a slower clearance of *Salmonella* from the systemic organs showed higher phagocytic activity and a higher number of phagocytes (macrophages) in the spleen. Genetic variation in the function of the phagocytes (macrophages) is also demonstrated in several other broiler lines (Qureshi and Miller, 1991).

Because we found some significant sire and dam effects and significant correlations between tests, the results indicate that these methods are useful to identify genetic differences within broiler lines that may be involved in disease resistance.

In conclusion, the present study demonstrates that two sets of two different broiler lines exhibit significant differences in the *S.* Enteritidis colonization of the intestine versus systemic organs. This difference seems to be related to differences in phagocyte function

and humoral immune responses. Moreover, the age that the chickens become infected played an important role for the ability of the chickens to clear the systemic infection. Further research will focus on the identification of the genetic variation within line to improve disease resistance of the broilers to *S.* Enteritidis.

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# Chapter 6

## GENETIC RESISTANCE TO *SALMONELLA* ENTERITIDIS CARRIER-STATE OF FIVE DIFFERENT GENETIC GROUPS OF MEAT-TYPE CHICKEN

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**SUMMARY**

*Salmonella* Enteritidis is a facultative intracellular pathogen that may cause disease in both human and animal. Genetic improvements of disease resistance may support control of this pathogen in animals of the food chain. Differences in *Salmonella* load in caecum content, spleen and liver between five genetic groups of chicken (two Old Dutch Breeds, group 1 and 2 and three broiler groups, group 3 to 5) are described in the current study. In total, 339 three week old birds were challenged with $5.5 \times 10^8$ c.f.u. *S.* Enteritidis and sacrificed at 4, 7 and 10 days post infection (DPI). Significant differences were found between groups according to *Salmonella* colonization in caecum content, spleen and liver. Overall, group 1 showed the highest colonization in all organs compared to the other groups. In contrast to the broiler groups, the Old Dutch Breeds were able to start clearing the infection from 4 to 10 DPI. Comparing all groups, group 2 appeared to be the group that was most potent to start clearing the infection and had low *Salmonella* load at 10 DPI in the organs. These results are useful in further search for identifying genes important in disease resistance.

## INTRODUCTION

Salmonellosis is a zoonotic disease that is a worldwide problem for both human safety and animal production. *Salmonella* enterica serotype Enteritidis (*S.* Enteritidis), a facultative intracellular bacterium, is one of the invasive intestinal bacterial pathogens that is capable of infecting a variety of hosts, resulting in manifestations like gastroenteritis, enteric fever and bacteremia. As most of these infections are food borne by food of animal origin (e.g. meat or eggs), genetic improvements of disease resistance in chicken may support control of this pathogen in the food chain (Goldberg and Rubin, 1988; Swartz, 2002).

An infection with *Salmonella* in human or animal usually starts after oral ingestion, followed by colonization in the gut. In mice and humans, it has been shown that *Salmonella* penetrates the mucosal epithelium of the small intestine using M cells after the bacteria attached to the intestinal epithelium (Neutra and Kraehenbuhl, 1992). The interaction between *Salmonella* and the epithelium stimulates the chemotaxis of phagocytic cells, like macrophages, that start to eliminate the bacterium (Desmidt et al., 1997; Henderson et al., 1999). However, studies have shown that *Salmonella* is able to survive within macrophages from many hosts (Buchmeier and Heffron, 1989; Abshire and Neidhardt, 1993; Alpuchearanda et al., 1995). These macrophages might play a role in the dissemination of *Salmonella* from the gut to various organs, such as liver and spleen (Carter and Collins, 1974; Popiel and Turnbull, 1985).

The majority of *Salmonella* infections in human are caused by consuming contaminated poultry products. Diseases like salmonellosis in animals could be controlled by hygiene strategies in husbandry supported by using vaccines and antibiotics. However, the extensive use of antibiotics and vaccines in humans or animals has been criticized in the last decade for different reasons. Pathogens may become resistant to antibiotics and residual antibiotics and attenuated vaccines in animal products for human consumption can be a potential danger. Another strategy to control an infection is breeding for animals that are genetically more resistant to various diseases or challenges. In addition, breeding has the advantage that the progress is structural and cumulative. Several studies have shown that considerable genetic variation exists between different chicken lines in response to *Salmonella* (Bumstead and Barrow, 1988; Bumstead and Barrow, 1993; Lindell et al., 1994; Protais et al., 1996; Duchet Suchaux et al., 1997; Beaumont et al., 1999; Kaiser and Lamont, 2001; Kramer et al., 2001). Therefore it is possible to enhance the immune responses by improvements of genetic disease resistance. This will lower the use of antibiotics and drug residues in food products, resulting in saver food products for human consumption.

The objective of this study was to investigate the differences in genetic resistance to *S.* Enteritidis between five meat-type chicken groups and whether these differences could be related to differences in immune parameters measured in a previous experiment

(Kramer et al., 2002). In that study, differences in natural resistance of several parameters of the innate and adaptive immune response important in a *Salmonella* infection were measured in seven meat-type chicken groups and significant differences were found between groups for most of the immune parameters measured. Together with the current study it is possible to compare the correlation in overall natural resistance with specific genetic resistance to a pathogen, *S.* Enteritidis between different groups.

## MATERIALS AND METHODS

*Animals*

Five genetically different meat-type chicken groups were used for this experiment (Table 6.1). They included two Old Dutch Breeds, group 1 (Barnevelder), obtained from IPC Dier, (Barneveld, The Netherlands) and the Barnevelderclub (Dronten, The Netherlands) and group 2 (Noord Hollandse Blauwe) obtained from IPC Dier and the Assendelfter and Noord-Hollandse Blauwenclub (Heiloo, The Netherlands). These Breeds have a relatively high mature body weight, although the weight of these chickens is considerable lower compared to mature, ad libitum fed broilers. Furthermore, three outbred broiler groups were included, group 3 (meat-type), 4 (meat-type but also selected for reproduction), and group 5 (offspring of the group 3 × group 4 cross). Groups 3, 4 and 5 were kindly provided by Hybro B.V. (Boxmeer, The Netherlands). In total, 339 animals were used of all groups (both male and female), as shown in Table 6.1.

**Table 6.1.** Number of chickens sacrificed per group on each day post infection (DPI), of sex and number of sires per group

| Group | DPI | | | Total | Sex | | | Sires |
|---|---|---|---|---|---|---|---|---|
| | T=4 | T=7 | T=10 | | Males | Females | Unknown | |
| 1 | 30 | 29 | 30 | 89 | 43 | 46 | | 6 |
| 2 | 18 | 21 | 18 | 57 | 31 | 26 | | 5 |
| 3 | 21 | 22 | 20 | 63 | 29 | 34 | | 11 |
| 4 | 18 | 21 | 23 | 62 | 30 | 31 | 1 | 9 |
| 5 | 21 | 24 | 23 | 68 | 30 | 38 | | 6 |
| Total | 108 | 117 | 114 | 339 | 163 | 175 | 1 | 37 |

After hatching, the chicken groups were housed separately in wire bottom cages. Birds were given feed and water ad libitum and were observed daily. After hatch, before the experiment started, it was determined that birds were free of *Salmonella* using standard bacteriological examination techniques. Within each group, half sibling progeny of different

sires were randomly chosen when sacrificed (Table 6.1). Birds were cared for in accordance with accepted procedures of the Dutch law on animal welfare. The animal experiment committee from the institute approved all experimental procedures applied to the animals.

*Infection with S. Enteritidis*

*S.* Enteritidis phage type 4 (nalidixic acid (Nal) resistant) was grown in buffered peptone water (BPW) overnight with shaking at 150 rpm. Three-week-old chickens were orally infected with 1.0 ml of a bacterial suspension containing $5.5 \times 10^8$ cfu *S.* Enteritidis.

*Necropsies*

Necropsies were performed at 4, 7 and 10 days post infection (DPI). The number of chicks of each group that were sacrificed at the different DPI is shown in Table 6.1. The number of infected animals (male or female) from each sire were allocated randomly over the three infection periods. At necropsy, before killing, total body weight of each chicken was measured and blood samples were taken, then chickens were killed by cervical dislocation. The liver, spleen and caecum were aseptically removed and kept at 4˚C until bacteriological examination.

*Bacteriological examination*

One gram of caecal content of each bird was homogenized in 9 ml BPW, serial diluted in BPW, and plated onto brilliant green agar with nalidixic acid (BGA-Nal$^+$) for quantitative *S.* Enteritidis determination. These BGA plates and the $10^{-1}$ dilution of the homogenate were incubated overnight at 37ºC. When at the lowest dilution no *Salmonella*e were found, the enriched $10^{-1}$ dilution was plated on BGA-Nal$^+$. Similarly, one g of liver or spleen tissue of each broiler was grounded separately in 9 ml BPW in a Colworth Stomacher 400 (A.J. Steward Company Ltd., London, UK) for 30 seconds, followed by selective plating with serial dilutions on BGA-Nal$^+$, as described above.

*Humoral responses*

Total antibody concentrations IgM, IgG and IgA were measured in plasma from animals sacrificed 10 DPI using a double antibody sandwich ELISA as previously described (Kramer et al., 2002). The known total antibody concentrations of the positive control were used to calculate total antibody concentrations of the samples.

Anti-*Salmonella* titers IgM and IgG were measured separately in plasma collected from animals sacrificed 10 DPI using an indirect ELISA based on *S.* Enteritidis LPS, as previously described (van Zijderveld et al., 1992).

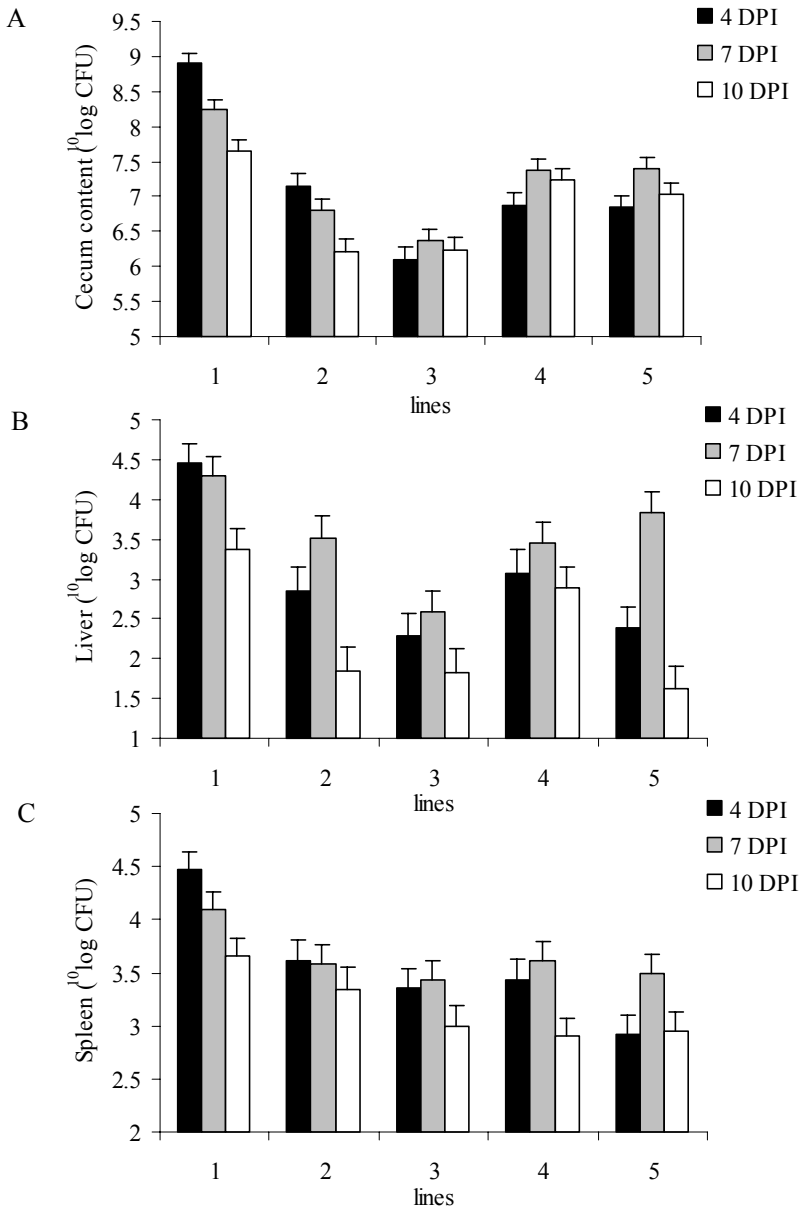


**Figure 6.1.** Means ± SEM of Salmonella colonization in the different organs (caecal content [6.1A], liver [6.1B], spleen [6.1C]) at the different days post infection (DPI) from chickens inoculated with S. Enteritidis ate the age of three weeks. Significant differences for caecum: group, DPI, sex, and group × DPI ($P < 0.001$), for liver: group, DPI ($P < 0.001$) and group × DPI ($P = 0.003$), and for spleen: group, and DPI ($P < 0.001$). Least significant differences (LSD) between groups for caecum content at 4 DPI LSD = 0.6, at 7 and 10 DPI LSD = 0.4, for liver at all DPI = 0.8, for spleen at all DPI LSD = 0.5

*Statistical analysis*

Due to the unbalanced design of this experiment, data were analyzed using Restricted Maximum Likelihood (REML) analysis (Genstat 5, Release 4.2, Fifth edition). A model with the factors for genetic groups, DPI, and sex together with random effects for sire was used for the analysis:

$$Y_{ijkl} = \mu + group_i + DPI_j + sex_k + sire_l + groupi \times DPI_{ij} + e$$

Where μ = overall mean, group = fixed effect for the i-th group, DPI = fixed effect for the j-th DPI, sex = fixed effect for k-th sex, sire = random effect for l-th sire, $group_i \times DPI_{ij}$ = the interaction between the i-th group and the j-th DPI and e = residual - not explained. Prior to analysis, the variables of the colony counting of caecum content, spleen and liver were transformed to logarithmic scale (10log) to normalize variances. Results shown in the tables or figure are predicted means, standard errors were calculated for the predicted means. All significant effects and interactions are given below each table or figure. The approximate Least Significant Difference (LSD) at $P < 0.05$ was calculated using predicted means and standard errors of the differences (SED). Two groups differed significantly when the difference between the means of two groups was higher than 1.96 × SED between those groups. The predicted means, together with the LSD were used to evaluate the data. The residuals from the REML analyses, being deviations of the various variables from a common adopted model, were used to calculate the correlations between the variables.

## RESULTS

*Bacteriological examination of caecum content*

The number of *S.* Enteritidis found in the caecum is presented in Figure 6.1A. A significant interaction was found for group × DPI ($P < 0.001$), and a significant effect was found for sex ($P < 0.001$). Figure 6.1A shows that group 1 had the highest *S.* Enteritidis counts at all DPI ($P < 0.05$). The number of *S.* Enteritidis found in the broiler groups was lower during the experiment compared to the Old Dutch Breeds. Mainly group 3 showed the lowest *S.* Enteritidis counts in the caecum during the whole experiment. Comparing the clearing activity of the different groups, results showed that the number of *S.* Enteritidis in caecum of group 1 and group 2 (the Old Dutch Breeds) decreased from 4 to 10 DPI ($P < 0.05$). In contrast, the *S.* Enteritidis load in caecum of the broiler groups increased between 4 to 7 DPI, where after the number decreased. At the last measurement in this experiment, at 10 DPI, group 2 and group 3 had significantly the lowest number of *S.* Enteritidis in the caecum content of all groups.

*Bacteriological examination of liver and spleen*

The results of the bacteriological examination of liver and spleen are shown in Figure 6.1B and 1C, respectively. For liver, a significant interaction was found for group × DPI ($P < 0.001$). Overall, group 1 had the highest *S.* Enteritidis counts in liver during the experiment and the *S.* Enteritidis load decreased from 4 DPI to 10 DPI ($P < 0.05$). In contrast, all other groups (groups 2 to 5) had an initial increase in *S.* Enteritidis counts from 4 to 7 DPI and after that a decrease from 7 to 10 DPI. Like for caecum, group 3 had the lowest bacterial load in liver during the experiment. At 10 DPI, significantly lower *S.* Enteritidis counts in liver were found in groups 2, 3 and 5 compared to groups 1 and 4 ($P < 0.05$).

The same pattern as for liver was found for *S.* Enteritidis counts in spleen. Significant effects for spleen were found for group and DPI ($P < 0.001$), no significant interaction was found. Like for caecum and liver, group 1 had the highest *S.* Enteritidis counts in spleen, and in this group the number of *S.* Enteritidis decreased during the experiment from 4 to 10 DPI ($P < 0.05$). The other groups did not decrease the number of *S.* Enteritidis as effective as group 1, these groups showed only a slight decrease in *S.* Enteritidis counts from 4 to 10 DPI. At the last measurement, at 10 DPI, the three broiler groups showed the lowest *S.* Enteritidis counts compared to the Old Dutch Breeds.

**Table 6.2.** Total antibody concentration in serum at 10 days post infection

| Group | Total Ig concentration (mg/ml) ± SEM | | | | | |
|---|---|---|---|---|---|---|
| | IgM[ab] | | IgG[ac] | | IgA[d] | |
| 1 | 0.67 | ± 0.01 | 5.12 | ± 0.09 | 0.35 | ± 0.01 |
| 2 | 0.64 | ± 0.01 | 4.83 | ± 0.09 | 0.36 | ± 0.01 |
| 3 | 0.66 | ± 0.01 | 4.99 | ± 0.09 | 0.35 | ± 0.01 |
| 4 | 0.61 | ± 0.01 | 4.69 | ± 0.08 | 0.34 | ± 0.01 |
| 5 | 0.63 | ± 0.01 | 4.74 | ± 0.09 | 0.34 | ± 0.01 |

[a]Group ($P < 0.001$)

[b]Least Significant Difference = 0.02

[c]Least Significant Difference = 0.16

[d]Least Significant Difference = 0.03

*Humoral immune response*

The total antibody titers and the specific *S.* Enteritidis antibody titers in the serum at 10 DPI are shown in respectively Tables 6.2 and 6.3. Significant effects for group ($P < 0.001$) were found for IgM and IgG for both the total and specific antibody titers. Similar patterns were found according to the differences between groups between the IgM and IgG responses for both the total and specific antibody titers. Group 1 had the highest

total and specific antibody titers for both IgM and IgG. In contrast, group 4 had the lowest antibody titers. No significant differences in effects for group were found for total IgA responses.

**Table 6.3.** Total antibody concentration in serum at 10 days post infection

| Group | *Salmonella* Ig titer ($^2$log antibody titer) | |
|---|---|---|
| | IgM[ab] | IgG[ac] |
| 1 | 10.2 ± 0.6 | 7.2 ± 0.8 |
| 2 | 8.8 ± 0.6 | 5.7 ± 0.9 |
| 3 | 9.6 ± 0.6 | 5.4 ± 0.8 |
| 4 | 7.1 ± 0.5 | 4.0 ± 0.7 |
| 5 | 8.6 ± 0.6 | 5.2 ± 0.8 |

[a]Group ($P < 0.001$)
[b]Least Significant Difference = 0.9
[c]Least Significant Difference = 1.4

*Body Weight*

Body weights of the chickens of different genetic groups were measured (results not shown). These results were comparable to results previously described (Kramer et al., 2002). Body weights of broiler chickens (around 1.3 kg at 30 days of age) were about three times as high compared to the Old Dutch Breeds (around 0.4 kg at 30 days of age). A significant interaction was found for group × DPI ($P < 0.001$) and an effect was found for sex ($P < 0.001$).

*Correlation between the tests and sire effects*

Various significant correlations between different tests were found (Table 6.4). The *S.* Enteritidis counts in the spleen and liver showed a highly significant correlation at all DPI ($P < 0.001$). Moreover, a significant negative correlation was found between the systemic infection in liver and the colony counts in the caecum content at 10 DPI ($P < 0.05$).
Interestingly, there tended to a negative correlation between weight and *S.* Enteritidis counts early after infection (4 DPI in caecum and liver bacterial counts, and 7 DPI in spleen counts).

Although not all correlations were significant, there tended to be a positive correlation between the colony counts in caecum and the total IgM, IgG and IgA responses, but not with the specific IgM and IgG responses. Moreover, a significant ($P = 0.003$) correlation was found between the *S.* Enteritidis counts in the spleen and the specific IgG responses and there tended ($P = 0.09$) to be a correlation between the *S.* Enteritidis counts and the specific IgM responses.

**Table 6.4.** Correlations between the different tests, after correction for the fixed effects[a]

| | Weight | Caecum 4 DPI | Caecum 7 DPI | Caecum 10 DPI | Liver 4 DPI | Liver 7 DPI | Liver 10 DPI | Spleen 4 DPI | Spleen 7 DPI | Spleen 10 DPI | IgM SE[b] | IgG SE[b] | IgM total[c] | IgG total[c] | IgA total[c] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Weight | 1.00 | | | | | | | | | | | | | | |
| Caecum 4 DPI | -0.15 | 1.00 | | | | | | | | | | | | | |
| Caecum 7 DPI | -0.03 | | 1.00 | | | | | | | | | | | | |
| Caecum 10 DPI | 0.09 | | | 1.00 | | | | | | | | | | | |
| Liver 4 DPI | -0.23 | -0.09 | | | 1.00 | | | | | | | | | | |
| Liver 7 DPI | -0.06 | | -0.11 | | | 1.00 | | | | | | | | | |
| Liver 10 DPI | 0.01 | | | -0.17 | | | 1.00 | | | | | | | | |
| Spleen 4 DPI | 0.14 | 0.02 | | | 0.25 | | | 1.00 | | | | | | | |
| Spleen 7 DPI | -0.27 | | -0.05 | | | 0.26 | | | 1.00 | | | | | | |
| Spleen 10 DPI | 0.06 | | | -0.11 | | | 0.25 | | | 1.00 | | | | | |
| IgM SE[b] | 0.00 | | | -0.10 | | | 0.02 | | | 0.16 | 1.00 | | | | |
| IgG SE[b] | 0.07 | | | -0.04 | | | 0.09 | | | 0.28 | 0.56 | 1.00 | | | |
| IgM total[c] | -0.16 | | | 0.14 | | | -0.04 | | | -0.04 | 0.29 | 0.11 | 1.00 | | |
| IgG total[c] | 0.00 | | | 0.22 | | | 0.00 | | | 0.11 | 0.16 | 0.13 | 0.30 | 1.00 | |
| IgA total[c] | -0.03 | | | 0.17 | | | -0.08 | | | 0.09 | 0.15 | -0.02 | 0.47 | 0.38 | 1.00 |

[a] $|r| > 0.16$ ($P < 0.10$), $|r| > 0.17$ ($P < 0.05$) and $|r| > 0.25$ ($P < 0.01$), fixed effects are group, DPI, and sex, random effect is sire
[b] IgM SE and IgG SE are the specific antibody responses at 10 DPI
[c] IgM total, IgG total and IgA total are the total antibody responses at 10 DPI

With respect to the antibody responses various significant correlations were found. The specific IgG and IgM responses showed a significant correlation ($P < 0.001$), and the total IgM, IgG and IgA responses also showed highly significant correlations ($P < 0.001$). Interestingly, a significant correlation was found between the total and the specific IgM immune responses ($P = 0.001$).

Although not significant, there tended to be a sire effect for weight, the specific IgM responses and the non-specific IgA responses ($P < 0.10$).

## DISCUSSION

Improving disease resistance to *Salmonella* in chicken can be used to diminish the number of contaminated animals in flocks or contaminated flocks, resulting in safer poultry-derived food products for human consumption. In this experiment the differences between five genetic groups of chicken with respect to *S.* Enteritidis colonization in various organs was investigated after an oral infection. Results indicate that the Old Dutch Breed group 1 had higher numbers of *S.* Enteritidis in the various organs tested on all DPI of the experiment compared to commercial broilers and the other Old Dutch Breed, group 2. Compared to the broilers, the clearing activity of both Old Dutch Breeds was higher, these groups were able to start clearing the infection more rapidly. In accordance with a high *S.* Enteritidis colonization in group 1, this group had the highest titers in both the specific and non-specific response. Overall, group 2 appeared to be the most resistant group, since group 2 is able to start clearing the infection in all organs from 4 DPI on, the *Salmonella* load is low at 10 DPI, and the total and specific antibody titers are moderate to high compared to other groups.

In a previous study these five groups were used to determine the differences between groups in their natural resistance, therefore, differences in several immune parameters of the innate and adaptive immune response important after an *Salmonella* infection were measured in vitro in meat-type chicken groups (Kramer et al., 2002). That study showed that meat-type chicken groups (groups 3, 4 and 5), have a lower natural resistance compared to the Old Dutch Breeds (groups 1 and 2). Especially group 2 showed good results in most of the immune traits measured. The current results underline the results of the previous study, in that group 2, which had the highest natural resistance, also appeared to be the most resistant group after a *S.* Enteritidis infection. Thus this suggest that measuring resistance by in vitro challenge is appropriate for phenotypic selection for *Salmonella* resistance.

Differences in clearing activity of *Salmonella* by the Old Dutch Breeds and broilers were compared to results found in the experiment about natural resistance, where the innate and adaptive immune responses were measured (Kramer et al., 2002). The main differences

in natural resistance measured between the Old Dutch Breeds and the broiler groups were the innate immune responses and the IgM and IgG responses. The Dutch Breeds had a higher innate immunity (mainly the nitric oxide production by leukocytes, but also in group 2 the rate of entrance and survival of *Salmonella* in splenic leukocytes). This is also supported by the positive correlation between the nitric oxide production and the survival of *Salmonella* in splenic leukocytes. So, this suggests that the first response of clearing *Salmonella* by the Dutch Breeds is due to the higher activity of the innate immune response. This seems likely, because the innate immune response is considered to be first line of immunological defense to pathogens. Later on in the infection, as the B cells are stimulated to produce the antibodies, the antibodies will also help to clear the infection by opsonizing the pathogen, where after it will be recognized by the phagocytes, and the infection will be cleared (Dietert et al., 1991; Janeway and Travers, 1997). This is also partially supported by the negative correlations between the entry and survival of *Salmonella* in splenic leukocytes and the humoral responses, because when the innate immune response is not sufficient, the adaptive immune response can be stimulated (Kramer et al., 2002). It has been described previously that the elimination of *S.* Enteritidis partially depends on the humoral, cell-mediated and on the innate immune responses (Arnold and Holt, 1995; Desmidt et al., 1998, Maskell et al., 1987). Thus, these and the current studies suggest that the first line of defense to start clearing a *Salmonella* infection is depended on the innate immunity, but later on in the infection, the adaptive immune response becomes important to have a full spectrum of immunological defense to combat the infection.

Groups 3 and 4 were investigated previously in an infection experiment, where one day old chicks were orally infected with *Salmonella*, and 7, 14 and 28 DPI the birds were investigated for their *Salmonella* counts and antibody titers (Kramer et al., 2001). Both lines were numbered similar in both the previous study (Kramer et al, 2001) and the current study. Results from the previous study showed that caecum colonization on various DPI was higher for group 3 than for group 4, while the *S.* Enteritidis counts in the liver and spleen tended to be higher for group 4 than for group 3. These results are in contrast to the results found in the present study, where group 4 not only had higher *S.* Enteritidis counts in caecum but also higher *S.* Enteritidis counts in liver and spleen compared to group 3. The antibody titers, however, are comparable in both experiments; in both experiments group 3 showed the highest antibody titers specific to *S.* Enteritidis. The differences in colonization between these two experiments can be due to several causes. First, the age of the birds can result in differences in response to an infection. Differences in *Salmonella* clearance between one day old chickens and three or four week old chickens have been described (Duchet Suchaux et al., 1995; Desmidt et al., 1997). Although these studies do not compare different lines, results showed that one day old chickens are much more susceptible to an infection than 3 or 4 week old chickens. Secondly, the different infection doses between the two experiments

might result in differences in response. Although in a study of Kaiser and Lamont (2001) no differences were found after inoculation of different doses in one day old chickens ($10^3$, $10^5$ and $10^7$ c.f.u./bird), together with the differences in age the dose might result in differences in colonization described in our studies.

Correlations were found between the different assays as depicted in Table 6.4. A significant correlation was found between the *Salmonella* counts in the caecum and the liver. Moreover, we found a significant correlation between the *Salmonella* counts in the liver and in the spleen. Other studies showed a lack of correlations between the *Salmonella* counts in caecum and spleen or liver (Kaiser and Lamont, 2001; Kramer et al., 2001). However, although the correlations were not significant, there tended to be an inverse relation between the severity of the caecal infection and the colonization in spleen and liver (Kramer et al., 2001). These and the current results show that the secondary infection in liver and spleen in chicken may be partially controlled by the same factors, like for example the penetration of the bacteria through the epithelium of the gut or the dissemination of the bacteria throughout the body. Another significant correlation was found between the specific and nonspecific IgM responses. This might lead to the conclusion that higher nonspecific IgM titers can give an indication about higher specific titers, like for *Salmonella* in this case. Furthermore, the IgM, IgG and IgA non-specific titers, were significant correlated to each other, and also the IgG and IgM specific titers showed a significant correlation, as we found in other studies (Kramer et al., 2001; Kramer et al., 2002).

At three weeks of age, the body weight of the broilers is at least 3 times as high compared to the body weight of the Dutch Breeds (Kramer et al., 2002), and all groups received the same *Salmonella* infection dose. Although the ratio of body weight and infection dose differed between the Old Dutch Breeds and the broilers, it was surprising that *Salmonella* counts in the different organs of group 2 were considerably lower than of group 1, comparable with the broilers. Interestingly, the correlation between weight and bacterial counts decreased from 4 to 10 DPI in caecum and liver. At 4 DPI there was a negative correlation with weight, which indicated that birds with a higher weight at 4 DPI had a higher *Salmonella* colonization in caecum and liver. The correlation of weight of bacterial load in caecum and liver at 7 and 10 DPI and splenic bacterial load at 10 DPI was not significant, which indicated that weight in a later stage of the infection is not related with bacterial load. This was as expected considering the results of colonization because the Old Dutch Breeds are able to start clearing the infection from 4 to 10 DPI, through which the bacterial load reaches the level of the broilers at 10 DPI. These results suggest that the level of *S.* Enteritidis colonization in broilers later on in infection is comparable to the Old Dutch Breeds. Although strong selection criteria have been implemented to breed broilers as they are nowadays, this implies that broilers are able to respond accurately to a *S.* Enteritidis infection, through which the infection remains moderate. However, the ability to clear the

infection is lower in the broiler groups compared to the Old Dutch Breeds, thus it could be possible that in the long term, the infection sustains, however this can not be concluded from this experiment, because the last measurement was at 10 DPI.

Differences in the susceptibility to infectious diseases among five genetically different groups were investigated considering *Salmonella* colonization in organs and several immune parameters. No significant sire effects were found, but this can be due to the small groups and structure of the experiment. There was variation within the groups however, based on the standard error of means. Sufficient genetic variation exists within and between groups to suggest that it is possible to select among birds or groups that exhibit reduced *Salmonella* content. Furthermore, it should be possible to select for genetic resistance based on phenotypic variation (as described in Kramer et al, 2002), since these results seemed to be correlated with infection level as described in the current study. It might even be possible to develop assays without sacrificing the animal by using for example blood cells to determine the susceptibility of an animal for infectious diseases. The current results can also be used to characterize what genes or loci are important in early disease resistance to *Salmonella* Enteritidis or other related pathogens like other serotypes of *Salmonella*, and those genetic markers can be used for selection in breeding programs.

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# Chapter 7.1

## ASSOCIATION OF TWELVE CANDIDATE GENE POLYMOPHISMS AND RESPONSE TO CHALLENGE WITH *SALMONELLA* ENTERITIDIS IN POULTRY

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**SUMMARY**

Breeding for disease resistance to *Salmonella* Enteritidis (*S.* Enteritidis) could be an effective approach to control *Salmonella* in poultry. The candidate gene approach is a useful method to investigate genes that are involved in genetic resistance. In this study, twelve candidate genes that are involved in the pathogenesis of *Salmonella* infection were investigated using five different genetic groups of meat-type chicken. The genes were natural resistance associated macrophage protein 1 (*NRAMP1*), inhibitor of apoptosis protein 1(*IAP-1*), prosaposin (*PSAP*), Caspase-1, inducible nitric oxide production (iNOS), interferon-gamma (*IFNγ*), interleukin-2 (*IL-2*), immunoglobulin light chain (IgL), ZOV3, and transforming growth factors β2, β3 and β4 (*TGFβ2*, *β3* and *β4*). In total, 117 birds of all groups were challenged with *S.* Enteritidis at the age of three weeks. In all birds at seven days post infection *S.* Enteritidis load in caecum content, spleen and liver were quantified. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assays were used to genotype all animals for each gene. Overall we found the most significant associations with caecum content, nine out of twelve genes showed a significant association (*NRAMP1, IAP-1, PSAP,* Caspase-1*, iNOS, IL-2, IgL, TGFβ2* and *β4*). For liver, five genes (*NRAMP1,* Caspase-1*, IL-2, IgL*, and *TGFβ4*) and for spleen, only one gene (*TGFβ3*) showed a significant association with *S.* Enteritidis load. By showing associations of twelve PCR-RFLP assays with *S.* Enteritidis load after a pathogen challenge, this study confirmed the polygenic nature of disease resistance to *S.* Enteritidis.

## INTRODUCTION

*Salmonella*-contaminated poultry products are a worldwide problem because of the pathogenicity for both humans and birds. The use of antibiotics and vaccines to control *Salmonella* infection in chickens has been criticized because of the possible development of antibiotic resistant bacteria, and the potential dangers of antibiotic and vaccine residues in animal-derived food products for human consumption. The enhancement of natural genetic resistance is, therefore, an alternative approach to control *Salmonella* in poultry. As proposed by Vint (1997), changes in the virulence of pathogens, concentration of poultry in larger production units, and failure of pathogen eradication in most commercial operations require genetic approaches to improve disease resistance. Genetic improvements of disease resistance will increase immune responses, and reduce the use of antibiotics and drug residues in food products.

Other studies have already demonstrated the polygenic nature of disease resistance to *Salmonella* Enteritidis (*S.* Enteritidis) in poultry (e.g. Bumstead & Barrow 1993; Lindell et al. 1994; Protais et al. 1996; Girard Santosuosso et al. 1998; Kramer et al. 2001). Candidate gene analysis is an efficient approach to dissect the genes that influence disease resistance to *Salmonella*, because much is known about the system in mouse and humans. So far, however, only a few candidate genes or regions with an association for *Salmonella* resistance were identified in chickens. Natural resistance associated macrophage protein 1 (*NRAMP1*) and TNC (a locus closely linked to the *LPS* gene in the mouse genome) showed a significant association on early resistance to *Salmonella* (Hu et al. 1997; Liu et al., 2002). Another region that was identified on chicken chromosome 5 showed a large effect on resistance to *Salmonella* (Mariani et al. 2001). Cotter et al. (1998) showed that the B-complex determined part of the differential resistance to *S.* Enteritidis. Furthermore, Lamont and co-workers (Lamont et al. 2002; Liu & Lamont, in preparation) described associations on early resistance to *Salmonella* in chicks with inhibitor of apoptosis protein 1 (*IAP-1*), prosaposin (*PSAP*) and Caspase-1. These studies illustrate that a lot of research has been done on genetic resistance to *Salmonella* in relation to pathogen load, survival and frequency of colonisation in chicks. However, limited studies report about gene association studies to *Salmonella* challenge in different genetic groups of meat-type chicken. Furthermore, few candidate genes have been investigated in multiple populations, a key feature in validating candidate genes. The objective of this study was to evaluate 12 candidate genes, using polymorphism assays developed by the group of S. J. Lamont (Zhou et al. 2001a; Lamont et al. 2002; Li et al. 2002; Liu et al. 2002; Liu & Lamont, in preparation; Malek and Lamont, 2002) and applied in their resource populations, in response to pathogen load after a *S.* Enteritidis challenge in genetic groups of meat-type chicken at the age of three weeks.

## MATERIALS AND METHODS

*Experimental animals*

Five different genetic groups of meat-type chicken were used for this experiment (Table 7.1.1). They included two Old Dutch Breeds, group 1 (Barnevelder), obtained from IPC Dier, (Barneveld, The Netherlands) and the Barnevelderclub (Dronten, The Netherlands) and group 2 (Noord Hollandse Blauwe) obtained from IPC Dier and the Assendelfter and Noord-Hollandse Blauwenclub (Heiloo, The Netherlands). These Breeds have a high mature body weight, although the weight of these chickens is much lower compared to mature, ad libitum fed broilers. Furthermore, three outbred broiler groups were included, group 3 (meat-type), group 4 (meat-type but also selected for reproduction), and group 5 (offspring of the group 3 × group 4 cross). Groups 3, 4 and 5 were kindly provided by Hybro B.V. (Boxmeer, The Netherlands). In total, 339 birds of all groups (both males and females) were challenged with *S.* Enteritidis at the age of three weeks. Animals were sacrificed at four, seven, and ten days post infection to quantify the *S.* Enteritidis load in caecum content, spleen and liver (Kramer et al., in preparation). For the current study, 117 animals were used that were sacrificed 7 days post infection, as shown in Table 7.1.1. Within each group, half-sibling progeny of different numbers of sires were selected (Table 7.1.1). After hatching, chicks were housed in wire-bottom cages, where each group was housed separately. Animals were given *ad libitum* access to feed and water and were observed daily. After hatch, it was determined that birds were free of *Salmonella*. All chickens were cared for in accordance with accepted procedures of the Dutch law on animal welfare.

**Table 7.1.1.** Number of animals and sires used for candidate gene analysis

| Clusters | Groups | Number of animals | Number of sires |
|---|---|---|---|
| Old Dutch Breeds | Group 1 | 29 | 6 |
| | Group 2 | 21 | 5 |
| Broilers | Group 3 | 22 | 8 |
| | Group 4 | 21 | 8 |
| | Group 5 | 24 | 6 |

*Salmonella pathogenic challenge and quantification of bacterial load*

*Salmonella* Enteritidis phage type 4 (nalidixic acid (Nal) resistant) was grown in buffered peptone water (BPW) overnight at 37ºC with shaking at 150 rpm. Three-week-old chickens were orally infected with 1.0 ml of a bacterial challenge suspension with $5.5 \times 10^8$ *S.* Enteritidis colony forming units, CFU (Kramer et al., in preparation). After measuring

total body weight, the birds were euthanized at 7 days post inoculation by cervical dislocation, and liver, spleen and caecum were removed for bacteriological examination.

*Bacteriological examination*

A one gram of caecal content of each bird was homogenized in 9 ml BPW, serial diluted in BPW, and plated onto brilliant green agar with nalidixic acid (BGA-$Nal^+$) for quantitative *S.* Enteritidis determination. These BGA plates and the $10^{-1}$ dilution of the homogenate were incubated at 37ºC. When at the lowest dilution no bacteria were found, the enriched $10^{-1}$ dilution was plated on BGA-$Nal^+$. Similarly, one gram of liver or spleen tissue of each broiler was ground separately in 9 ml BPW in a Colworth Stomacher 400 (A.J. Steward company Ltd., London, UK) for 30 seconds, followed by selective plating with serial dilutions on BGA-$Nal^+$, as described above.

*Isolation of DNA and polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP)*

Chicken genomic DNA was isolated from venous blood collected in EDTA (using QIAamp DNA mini Kit, Westburg, Leusden, The Netherlands). All candidate genes were analyzed in the Iowa resource populations and were described previously (Zhou et al. 2001a; Li et al. 2002; Liu et al., 2002; Liu and Lamont, in preparation; Malek and Lamont, 2002). Briefly, PCR amplifications were carried out in 25 µl reaction volumes containing approximately 25 ng genomic DNA, 0.8 µM of each primer (Table 7.1.2), 200 µM of each dNTP, 1 unit of *Taq* DNA polymerase, 2.5 µl of 10 × PCR reaction Buffer, and $MgCl_2$ (Table 7.1.2). The PCR was performed for 36 three-step cycles at 94˚C for 45 seconds, optimal annealing temperature (Table 7.1.2) for 1 minute and 72˚C for 1 minute. For sequence analyses, amplified products were purified using a MICROCON® centrifugal filter (Milipore Corporation, Bedford, MA 01730). Nucleotide sequencing was performed by the DNA Sequencing and Synthesis Facility (Iowa State University, Ames, IA 20011). For each gene at least three different randomly selected animals were sequenced to confirm the single nucleotide polymorphism (SNP) that was found in the Iowa resource populations.

For PCR-RFLP analyses, restriction digestion of each single nucleotide polymorphism (SNP), a total final reaction volume of 20 µl was used (Table 7.1.3) and incubated for 4 hours to overnight. Separation was by electrophoresis through 1.5% to 3.0% agarose gels. For each gene, all animals of each group were genotyped.

*Data analysis*

Restricted Maximum Likelihood (REML, Genstat 5, Release 4.2, Fifth edition) analysis was used to analyze the association between the different genes and the

**Table 7.1.2.** Primer design for PCR-RFLP analysis of 12 candidate genes on genomic DNA

| Gene | GenBank accession # | Primer | Sequence | PCR product | Annealing Tm / Mg2+ for PCR amplification | Reference |
|---|---|---|---|---|---|---|
| *NRAMP* | U40598 | NR7F<br>NR7R | 5'-GGC GTC ATC CTG GGC TGC TAT-3'<br>5'-AGA CCG TTG CGA AGT CAT GC-3' | 801 | 64°C / 1.5 mM | (Lamont et al., 2002; Liu et al., 2002) |
| *IAP-1* | AF008592 | IAP1E1F<br>IAP1E1R | 5'-TCA CCA TCT CTA CGT TCC AT-3'<br>5'-CAT TGA AAC TTG GTT GGT CT-3' | 394 | 62°C / 1.5 mM | (Lamont et al., 2002; Liu and Lamont, in preparation) |
| *PSAP* | AB003471 | PSAP5F<br>PSAP5R | 5'-GGACATTTGTGCCATGGTT-3'<br>5'-GGCTTTCTCCACAGTTTCCA-3' | 112 | 55°C / 1.5 mM | (Lamont et al., 2002; Liu and Lamont, in preparation) |
| Caspase-1 | AF031351 | CASPF1<br>CASPR1 | 5'-CCA TGC TTG GGC TCT CAG TG-3'<br>5'-GGT CCC GCA GAT CCC AGT G-3' | 1070 | 60°C / 1.5 mM | (Liu and Lamont, in preparation) |
| *iNOS* | U46504 | INF7F<br>INR4 | 5'-CCA ATA AAA GTA GAA GCG A-3'<br>5'-CTC TTC CAG GAC CTC CA-3' | 491 | 50°C / 1.5 mM | (Malek and Lamont, 2002) |
| *IFNγ* | Y079221 | IFNpF2<br>IFNpR2 | 5'-ATT CTG ATG TCT GCC AC-3'<br>5'-GGC TTA GGC ATA CTC TTA-3' | 129 | 57°C / 1.5 mM | (Zhou et al., 2001a) |
| *IL-2* | AJ224516 | IL2PF<br>IL2PR | 5'-TGC TTT TAA CCG TCT TTG-3'<br>5'-GAT GCT CCA TAA GCT GTA GT-3' | 659 | 59°C / 1.5 mM | (Zhou et al., 2001a) |
| *IgL* | M24403 | IgLCF<br>IgLCR | 5'-TTT ATA CCC GCG TCC TTC-3'<br>5'-GGG AAA TAC TGG TGA TAG GTG-3' | 354 | 59°C / 1.5 mM | (Heltemes et al., 1997; Zhou et al., 2001a) |
| *ZOV3* | D16151 | ZOV3RNR<br>ZOV3NR | 5'-GCT TGG ACC TGG TAT ATG AC -3'<br>5'-CAT TCA GTA TTT TCA GAT GGG -3' | 320 | 52°C / 1.5 mM | (Zhou et al., 2001b) |
| *TGFβ2* | AF459831 | TGFB2PNF<br>TGFB2PNR | 5'-GCC ATA GGT TCA GTG CAA G-3'<br>5'-TGA CAG AAG CTC TCA AGC C-3' | 284 | 52°C / 1.5 mM | (Li et al., 2002) |
| *TGFβ3* | AF459835 | TGFB3E4F<br>TGFB3E4R | 5'-CGG CCT GGA AAT CAG CAT AC-3'<br>5'-GAA GCA GTA GTT GGT ATC CAG-3' | 1078 | 56°C / 1.5 mM | (Li et al., 2002) |
| *TGFβ4* | AF459837 | TGFB4NF<br>TGFB4NR | 5'-GGG GTC TTC AAG CTG AGC GT-3'<br>5'-TTG GCA ATG CTC TGC ATG TC-3' | 230 | 56°C / 1.0 mM | (Li et al., 2002) |

*S.* Enteritidis counts in caecum, liver and spleen. To enlarge the power of the analysis, the Old Dutch Breeds were clustered together, and the three broiler groups were clustered together. This was because of the similarities in genetic background of the different groups in the two clusters and the way in which these clusters of different groups responded to *S.* Enteritidis infection (Kramer et al., in preparation). Each cluster was analyzed separately for each gene. To improve the homogeneity of the parameters, the *S.* Enteritidis counts from the organs were log transformed ($^{10}$log).

**Table 7.1.3.** RFLP analysis of candidate genes

| Gene | SNP location | Restriction enzyme | Incubation |
|---|---|---|---|
| *NRAMP1* | $Ser^{379}$ (C → T) | *Sac* I (5 unit) | 37 °C |
| *IAP-1* | $Ala^{157}$ (G → A) | *Bgl* I (5 unit) | 37 °C |
| *PSAP* | $Gly^{271}$ (G → A) | *Tfi* I (5 unit) | 65 °C |
| Caspase-1 | -368 bp of 5' flanking region (C → T) | *Hsp92* II (5 unit) | 37 °C |
| *iNOS* | Intron (C → T) | *Alu* I (6 unit) | 37 °C |
| *IFNγ* | -318 bp of 5' flanking region (A → G) | *Tsp509* I (2 unit) | 65 °C |
| *IL-2* | -425 bp of 5' flanking region (A → G) | *Mnl* I (5 unit) | 37 °C |
| *IgL* | 60 bp upstream of octamer sequence (A → G) | *Sau96* I (5 unit) | 37 °C |
| *ZOV3* | $Val^{216}$→ $Leu^{216}$ (A → G) | *Sna* BI (2.5 unit) | 37 °C |
| *TGFβ2* | -1667 bp of 5' flanking region (C → T) | *Rsa* I (5 unit) | 37 °C |
| *TGFβ3* | -171 bp of exon 5 (T → G) | *Bsr* I (6 unit) | 37 °C |
| *TGFβ4* | $Glu^{210}$→ $Asp^{210}$ (A → C) | *Mbo* II (1.5 unit) | 37 °C |

The statistical model used:

$$\text{Model 1: } Y_{ij} = \mu + \text{gene} + \text{group}_i + \text{sex}_j + \text{sire} + \text{group}_i \times \text{gene}_i + e_{ij}$$

Where μ = overall mean, gene = fixed effect each candidate gene, group = fixed effect for the i-th group, sire = random effect sire, sex = fixed effect for j-th sex, and $\text{group}_i \times \text{gene}_i$ = the interaction between the i-th group and the gene, and e = residual. Furthermore, if a group was homozygous for a gene, that group was excluded from the cluster in the analysis of that gene.

The percentage of the effect of each gene accounted for the phenotypic variation of caecum, liver or spleen bacterial load was calculated. Therefore, Model 1 was taken as a full model, and a reduced model (Model 2) was build with only group and sex (fixed effects) and sire (random effect) as explanatory variables.

**Table 7.1.4.** Effect of 12 candidate genes on bacterial load in caecum ($^{10}$Log CFU) after REML analysis (P values and predicted means ± standard error of means (N))

| Gene[1] | Group[2] | *P*-values | $R^2$ (%) | Genotype | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | 11 | | 12 | | 22 | |
| *NRAMP* | Old Dutch Breeds | 0.008 | 6 | 7.45 ± 0.30 | (9) | 7.97 ± 0.18 | (18) | 7.57 ± 0.17 | (23) |
| | Broilers | <0.001 | 6 | 7.27 ± 0.06 | (50) | 7.13 ± 0.14 | (15) | 6.48 ± 0.42 | (2) |
| *IAP-1* | Old Dutch Breeds | 0.17 | 0 | 6.66 ± 0.43 | (5) | 7.16 ± 0.31 | (11) | 6.95 ± 0.45 | (5) |
| | Broilers | <0.001 | 7 | 6.29 ± 0.21 | (4) | 7.34 ± 0.09 | (27) | 7.22 ± 0.08 | (36) |
| *PSAP* | Old Dutch Breeds | 0.023 | 5 | 7.56 ± 0.18 | (16) | 7.51 ± 0.19 | (17) | 8.01 ± 0.18 | (17) |
| | Broilers | 0.018 | 8 | 7.17 ± 0.08 | (25) | 7.20 ± 0.08 | (31) | 7.44 ± 0.13 | (11) |
| Caspase-1 | Old Dutch Breeds | Homozygous (CC) | | | | | | | |
| | Broilers | <0.001 | 7 | 6.92 ± 0.17 | (18) | 7.15 ± 0.08 | (35) | 7.31 ± 0.16 | (14) |
| *iNOS* | Old Dutch Breeds | <0.001 | 2 | 7.75 ± 0.14 | (36) | 7.38 ± 0.29 | (12) | 7.74 ± 0.52 | (2) |
| | Broilers | 0.12 | 1 | 7.28 ± 0.07 | (46) | 7.14 ± 0.13 | (20) | 7.28 ± 0.44 | (1) |
| *IFNγ* | Old Dutch Breeds | 0.15 | 34 | 6.88 ± 0.49 | (3) | 6.67 ± 0.30 | (13) | 8.07 ± 0.43 | (5) |
| | Broilers | 0.38 | 1 | 6.95 ± 0.44 | (3) | 7.25 ± 0.15 | (12) | 7.22 ± 0.06 | (52) |
| *IL-2* | Old Dutch Breeds | 0.08 | 7 | 7.60 ± 0.14 | (37) | 7.81 ± 0.30 | (10) | 8.18 ± 0.46 | (3) |
| | Broilers | <0.001 | 1 | 7.64 ± 0.25 | (3) | 7.16 ± 0.11 | (25) | 6.54 ± 0.10 | (18) |
| *IgL* | Old Dutch Breeds | 0.13 | 2 | 7.73 ± 0.13 | (39) | 7.80 ± 0.40 | (9) | 8.45 ± 0.53 | (2) |
| | Broilers | 0.05 | 1 | 7.25 ± 0.06 | (54) | 7.19 ± 0.19 | (12) | 7.11 ± 0.44 | (1) |
| *ZOV3* | Old Dutch Breeds | 0.07 | 0 | 7.37 ± 0.41 | (6) | 7.02 ± 0.45 | (6) | 6.69 ± 0.31 | (9) |
| | Broilers | Homozygous (AA) | | | | | | | |
| *TGFβ2* | Old Dutch Breeds | Homozygous (TT) | | | | | | | |
| | Broilers | 0.001 | 2 | 7.42 ± 0.22 | (8) | 7.26 ± 0.09 | (32) | 7.30 ± 0.10 | (27) |
| *TGFβ3* | Old Dutch Breeds | 0.16 | 4 | 6.76 ± 0.22 | (15) | 7.72 ± 0.43 | (4) | 7.07 ± 0.61 | (2) |
| | Broilers | 0.75 | 0 | 6.57 ± 0.09 | (20) | 6.66 ± 0.29 | (2) | | (0) |
| *TGFβ4* | Old Dutch Breeds | <0.001 | 1 | 6.82 ± 0.22 | (11) | 7.94 ± 0.29 | (10) | 7.95 ± 0.38 | (28) |
| | Broilers | 0.030 | 2 | | (0) | 7.21 ± 0.18 | (9) | 7.25 ± 0.06 | (58) |

[1]For *NRAMP1*, Caspase-I, *iNOS*, and *TGFβ2* the genotype 11 = CC, 12 = CT and 22 = TT, for *IAP-1, PSAP, IFNγ, IL-2, IgL* and *ZOV3* the genotype 11 = AA, 12 = AG and 22 = GG, and for *TGFβ3* and TGFβ4 the genotype 11 = AA, 12 = AC and 22 = CC.

[2]Group 1 homozygous for *IAP-1* = GG, *IFNγ*= GG, *TGFβ3* = AA, and *ZOV3* = AA, group 4 homozygous for *IL-2* = AA and *TGFβ3* = AA, and group 5 homozygous for *TGFβ3* = AA

Model 2: $Y_{ij} = \mu + group_i + sex_j + sire + e_{ij}$

Then the correlation ($R^2$) was calculated of the phenotypic trait with the residual of the full model $Y_{ij} - e_{ij}$ (Model 1) and $R^2$ of the phenotypic trait with the residual of reduced model $Y_{ij} - e_{ij}$ (Model 2). Then, the percentage phenotypic variation accounted for each gene was calculated as 100% × ($R^2$ of model 1 minus $R^2$ of model 2). Because sire was taken as a random effect, the percentage phenotypic variation will not be absolutely correct, the value can be underestimated. However, it gives an indication about the effect of the gene accounted for the phenotypic variation.

## RESULTS

*Genomic sequence characterization*

All PCR-RFLP assays used in the current study were developed at Iowa State University using the Iowa resource populations [Iowa *Salmonella* Response Resource Population (ISRRP) and Iowa Antibody Kinetics Resource Population (IAKRP; Zhou et al. 2001a; Li et al. 2002; Liu et al., 2002; Liu & Lamont, in Preparation)]. In the current study, unrelated Dutch meat-type chicken groups were used to investigate associations of candidate genes with *S.* Enteritidis resistance. All SNPs characterized by PCR-RFLP assays previously were present in these groups. Although a few new SNPs were found, sequence analysis showed that the majority of the polymorphic sites found in 12 candidate gene sequences of the Dutch population were already described in the Iowa resource population. Only one polymorphism described previously by Zhou and co-workers (2001a) on the *IgL* gene was changed, but on the same position. In the current study an A/G mutation was found 60 bp upstream of the octamer sequence, and Zhou et al. (2001a) described a T/C mutation on the same position.

The associations of the candidate genes with *S.* Enteritidis load in caecum, liver and spleen are summarized in Table 7.1.4, 7.1.5 and 7.1.6, respectively. Overall, most significant associations with the 12 candidate genes were found with *S.* Enteritidis counts in caecum (in both Old Dutch Breeds and broilers).

*Association of the genotypes with Salmonella response with the candidate genes*

The silent mutation located at $Ser^{379}$ in *NRAMP1* (Table 7.1.3) showed a significant association ($P < 0.01$) with caecum *S.* Enteritidis load in both clusters, Old Dutch Breeds and broilers, and a significant association ($P = 0.05$) with liver *S.* Enteritidis load in broilers. The genotype "CC" was correlated with a higher *S.* Enteritidis load in caecum and liver in broilers and hence the genotype "TT" was correlated with a lower *S.* Enteritidis load in broilers (Table 7.1.4 and 7.1.5).

**Table 7.1.5.** Effect of 12 candidate genes on bacterial load in liver ($^{10}$Log CFU) after REML analysis (*P* values and predicted means ± standard error of means (N))

| Gene[1] | Group[2] | *P*-values | $R^2$ (%) | Genotype | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | 11 | | 12 | | 22 | |
| *NRAMP* | Old Dutch Breeds | 0.25 | 8 | 3.91 ± 0.37 | (9) | 3.64 ± 0.22 | (18) | 3.93 ± 0.21 | (23) |
| | Broilers | 0.05 | 2 | 3.19 ± 0.21 | (50) | 2.68 ± 0.43 | (15) | 2.37 ± 1.20 | (2) |
| *IAP-1* | Old Dutch Breeds | 0.26 | 6 | 3.14 ± 0.37 | (5) | 3.44 ± 0.28 | (11) | 3.24 ± 0.40 | (5) |
| | Broilers | 0.43 | 0 | 2.85 ± 0.58 | (4) | 2.90 ± 0.25 | (27) | 3.16 ± 0.21 | (36) |
| *PSAP* | Old Dutch Breeds | 0.60 | 11 | 3.77 ± 0.23 | (16) | 3.77 ± 0.24 | (17) | 3.82 ± 0.22 | (17) |
| | Broilers | 0.69 | 2 | 3.02 ± 0.26 | (25) | 3.29 ± 0.25 | (31) | 2.87 ± 0.39 | (11) |
| Caspase-1 | Old Dutch Breeds | Homozygous (CC) | | | | | | | |
| | Broilers | 0.003 | 3 | 2.98 ± 0.49 | (18) | 3.47 ± 0.24 | (35) | 3.10 ± 0.46 | (14) |
| *iNOS* | Old Dutch Breeds | 0.15 | 2 | 3.77 ± 0.19 | (36) | 3.75 ± 0.38 | (12) | 3.60 ± 0.70 | (2) |
| | Broilers | 0.93 | 0 | 3.26 ± 0.21 | (46) | 3.17 ± 0.36 | (20) | 2.94 ± 1.21 | (1) |
| *IFNγ* | Old Dutch Breeds | 0.19 | 14 | 3.77 ± 0.48 | (3) | 3.29 ± 0.26 | (13) | 3.04 ± 0.41 | (5) |
| | Broilers | 0.26 | 16 | 4.22 ± 1.19 | (3) | 2.82 ± 0.41 | (12) | 3.11 ± 0.23 | (52) |
| *IL-2* | Old Dutch Breeds | 0.029 | 5 | 3.81 ± 0.17 | (37) | 3.24 ± 0.38 | (10) | 3.87 ± 0.59 | (3) |
| | Broilers | 0.002 | 6 | 2.56 ± 0.68 | (3) | 3.16 ± 0.32 | (25) | 2.41 ± 0.27 | (18) |
| *IgL* | Old Dutch Breeds | 0.22 | 6 | 3.76 ± 0.15 | (39) | 3.60 ± 0.49 | (9) | 3.03 ± 0.65 | (2) |
| | Broilers | 0.046 | 3 | 3.03 ± 0.19 | (54) | 3.25 ± 0.51 | (12) | 5.38 ± 1.21 | (1) |
| *ZOV3* | Old Dutch Breeds | 0.08 | 0 | 3.24 ± 0.35 | (6) | 3.07 ± 0.39 | (6) | 3.58 ± 0.27 | (9) |
| | Broilers | Homozygous (AA) | | | | | | | |
| *TGFβ2* | Old Dutch Breeds | Homozygous (TT) | | | | | | | |
| | Broilers | 0.40 | 0 | 2.94 ± 0.63 | (8) | 3.20 ± 0.25 | (32) | 3.27 ± 0.29 | (27) |
| *TGFβ3* | Old Dutch Breeds | 0.50 | 2 | 3.45 ± 0.21 | (15) | 2.99 ± 0.40 | (4) | 3.05 ± 0.54 | (2) |
| | Broilers | 0.18 | 0 | 2.45 ± 0.27 | (20) | 1.44 ± 0.75 | (2) | | (0) |
| *TGFβ4* | Old Dutch Breeds | 0.044 | 1 | 3.30 ± 0.29 | (11) | 3.85 ± 0.38 | (10) | 3.44 ± 0.49 | (28) |
| | Broilers | 0.046 | 6 | | (0) | 2.73 ± 0.50 | (9) | 3.20 ±0.20 | (58) |

[1]For *NRAMP1*, Caspase-I, *iNOS*, and *TGFβ2* the genotype 11 = CC, 12 = CT and 22 = TT, for *IAP-1, PSAP, IFNγ, IL-2, IgL* and *ZOV3* the genotype 11 = AA, 12 = AG and 22 = GG, and for *TGFβ3* and TGFβ4 the genotype 11 = AA, 12 = AC and 22 = CC.

[2]Group 1 homozygous for *IAP-1* = GG, *IFNγ*= GG, *TGFβ3* = AA, and *ZOV3* = AA, group 4 homozygous for *IL-2* = AA and *TGFβ3* = AA, and group 5 homozygous for *TGFβ3* = AA

The polymorphisms of *IAP-1* (silent mutation located at $Ala^{157}$, Table 7.1.3), *PSAP* (silent mutation located at $Gly^{271}$, Table 7.1.3) and Caspase-1 (polymorphism in promoter region, Table 7.1.3) showed a significant association with *S.* Enteritidis load in caecum in broilers. Moreover, *PSAP* also showed an association with *S.* Enteritidis load in caecum in Old Dutch Breeds, and Caspase-1 showed a significant association with liver *S.* Enteritidis load in broilers. The genotype "GG" tended to have a higher *S.* Enteritidis load in caecum than the genotype "AA" for *IAP-1* and *PSAP* (Table 7.1.4). For Caspase-1, all Old Dutch Breeds were homozygous ("CC"). The genotype "TT" of Caspase-1 in broilers showed higher *S.* Enteritidis counts in caecum than the genotype "CC" (Table 7.1.4).

Most animals of all groups had the "CC" or "CT" genotype of the polymorphism found in *iNOS*, only three animals out of 117 tested had the genotype "TT" (Table 7.1.4-7.1.6). Significant associations were found with bacterial counts in caecum in Old Dutch Breeds ($P < 0.001$, Table 7.1.4), and within the broiler groups *iNOS* tended to have an effect in caecum *S.* Enteritidis load ($P = 0.12$, Table 7.1.4). Comparing the "CC" genotype with the "CT" genotype in all organs, it appeared that the "CC" genotype was associated with a higher *S.* Enteritidis load in caecum (Table 7.1.4).

For interferon-gamma (*IFNγ*), all animals of group 1 were homozygous ("GG") for the promoter region, and only 6 of 117 birds total had the genotype "AA". Although no significant associations were found for bacterial load in the organs, there tended ($P = 0.12$) to be an association with the *S.* Enteritidis load in spleen with the broiler groups. Animals of group 4 were homozygous "AA" for the SNP investigated in the promoter region, -425 bp of the 5' flanking region of interleukin–2 (*IL-2*). Furthermore, group 3 only had genotype "AG" or "GG", and group 5 only "AA" or "AG". An association of *IL-2* with *S.* Enteritidis load in caecum and liver was found for both Old Dutch Breeds ($P < 0.08$) and broilers ($P < 0.002$; Table 7.1.4). Because of the unequal distribution of the various genotypes of *IL-2* and *IFNγ* between the groups and the clusters it is not possible to make any suggestions about the genotype that would be associated with a higher *S.* Enteritidis load (Table 7.1.4 – 7.1.6).

The polymorphism in the promoter region of immunoglobulin light chain (*IgL*) had an association ($P < 0.05$) with *S.* Enteritidis counts in caecum and liver (Table 7.1.4 and 7.1.5). The SNP $Val^{216}$ / $Leu^{216}$ found in *ZOV3* tended to be associated with caecum ($P = 0.07$) and liver ($P = 0.08$) *S.* Enteritidis load of the Old Dutch Breed, group 2, as all other groups were homozygous. Results suggest that the genotype "AA" of *ZOV3* is associated with a higher *S.* Enteritidis load in caecum, but somewhat lower in liver and spleen (Table 7.1.4 – 7.1.6).

Both Dutch Breeds were homozygous for *TGFβ2*. A significant association ($P = 0.001$) for the polymorphism located on –1667 bp of 5' flanking region of *TGFβ2* was found for caecum *S.* Enteritidis load in broilers. Results suggest that the genotype "CC" is associated with a higher *S.* Enteritidis load in caecum, but somewhat lower in liver and

**Table 7.1.6.** Effect of 12 candidate genes on bacterial load in spleen ($^{10}$Log CFU) after REML analysis (*P* values and predicted means ± standard error of means (N))

| Gene[1] | Group[2] | *P*-values | $R^2$ (%) | Genotype | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | 11 | | 12 | | 22 | |
| *NRAMP* | Old Dutch Breeds | 0.30 | 14 | 3.30 ± 0.24 | (9) | 3.74 ± 0.17 | (18) | 3.70 ± 0.17 | (23) |
| | Broilers | 0.10 | 11 | 3.40 ± 0.08 | (50) | 3.04 ± 0.19 | (15) | 3.99 ± 0.55 | (2) |
| *IAP-1* | Old Dutch Breeds | 0.52 | 0 | 3.69 ± 0.20 | (5) | 3.31 ± 0.14 | (11) | 3.51 ± 0.21 | (5) |
| | Broilers | 0.72 | 3 | 3.37 ± 0.28 | (4) | 3.31 ± 0.12 | (27) | 3.40 ± 0.10 | (36) |
| *PSAP* | Old Dutch Breeds | 0.68 | 6 | 3.81 ± 0.15 | (16) | 3.65 ± 0.16 | (17) | 3.78 ± 0.15 | (17) |
| | Broilers | 0.87 | 8 | 3.32 ± 0.11 | (25) | 3.41 ± 0.11 | (31) | 3.47 ± 0.17 | (11) |
| Caspase-1 | Old Dutch Breeds | Homozygous (CC) | | | | | | | |
| | Broilers | 0.44 | 5 | 3.35 ± 0.24 | (18) | 3.48 ± 0.11 | (35) | 3.46 ± 0.22 | (14) |
| *iNOS* | Old Dutch Breeds | 0.67 | 4 | 3.71 ± 0.12 | (36) | 4.08 ± 0.22 | (12) | 3.70 ± 0.41 | (2) |
| | Broilers | 0.78 | 4 | 3.38 ± 0.09 | (46) | 3.41 ± 0.16 | (20) | 3.07 ± 0.57 | (1) |
| *IFNγ* | Old Dutch Breeds | 0.86 | 1 | 3.46 ± 0.28 | (3) | 3.46 ± 0.14 | (13) | 3.44 ± 0.23 | (5) |
| | Broilers | 0.12 | 10 | 2.59 ± 0.55 | (3) | 3.37 ± 0.18 | (12) | 3.39 ± 0.08 | (52) |
| *IL-2* | Old Dutch Breeds | 0.42 | 10 | 3.62 ± 0.14 | (37) | 4.14 ± 0.22 | (10) | 3.91 ± 0.32 | (3) |
| | Broilers | 0.92 | 0 | 3.22 ± 0.37 | (3) | 3.35 ± 0.17 | (25) | 3.30 ± 0.15 | (18) |
| *IgL* | Old Dutch Breeds | 0.74 | 0 | 3.71 ± 0.12 | (39) | 3.87 ± 0.28 | (9) | 3.79 ± 0.40 | (2) |
| | Broilers | 0.62 | 8 | 3.40 ± 0.08 | (54) | 3.01 ± 0.24 | (12) | 3.01 ± 0.56 | (1) |
| *ZOV3* | Old Dutch Breeds | 0.38 | 0 | 3.28 ± 0.20 | (6) | 3.46 ± 0.22 | (6) | 3.56 ± 0.15 | (9) |
| | Broilers | Homozygous (AA) | | | | | | | |
| *TGFβ2* | Old Dutch Breeds | Homozygous (TT) | | | | | | | |
| | Broilers | 0.88 | 7 | 3.36 ± 0.29 | (8) | 3.38 ± 0.11 | (32) | 3.51 ± 0.13 | (27) |
| *TGFβ3* | Old Dutch Breeds | <0.001 | 30 | 3.57 ± 0.09 | (15) | 2.86 ± 0.17 | (4) | 3.70 ± 0.23 | (2) |
| | Broilers | 0.49 | 3 | 3.26 ± 0.16 | (20) | 3.64 ± 0.50 | (2) | | (0) |
| *TGFβ4* | Old Dutch Breeds | 0.19 | 1 | 3.41 ± 0.22 | (11) | 3.75 ± 0.23 | (10) | 3.52 ± 0.28 | (28) |
| | Broilers | 0.37 | 7 | | (0) | 3.44 ± 0.23 | (9) | 3.39 ± 0.07 | (58) |

[1]For *NRAMP1*, Caspase-I, *iNOS*, and *TGFβ2* the genotype 11 = CC, 12 = CT and 22 = TT, for *IAP-1, PSAP, IFNγ, IL-2, IgL* and *ZOV3* the genotype 11 = AA, 12 = AG and 22 = GG, and for *TGFβ3* and TGFβ4 the genotype 11 = AA, 12 = AC and 22 = CC.

[2]Group 1 homozygous for *IAP-1* = GG, *IFNγ*= GG, *TGFβ3* = AA, and *ZOV3* = AA, group 4 homozygous for *IL-2* = AA and *TGFβ3* = AA, and group 5 homozygous for *TGFβ3* = AA

spleen (Table 7.1.4 – 7.1.6). Only group 2 and 3 were heterozygous for the polymorphism of *TGFβ3* at -171 bp of exon 5. A significant association for *TGFβ3* was found of spleen *S.* Enteritidis load for group 2, as group 1 was homozygous for "AA". The amino acid change from $Glu^{210}$ to $Asp^{210}$ of *TGFβ4* resulted in significant associations of Old Dutch Breeds and broilers with caecum and liver *S.* Enteritidis counts ($P < 0.05$). Comparing the various genotypes, it appeared that the genotype "CC" had a higher *S.* Enteritidis load in caecum and liver than chickens with the genotype "AA" for *TGFβ4* (Table 7.1.5).

*Phenotypic variation*

The phenotypic variation of each gene is given as $R^2$ in Table 7.1.4, 7.1.5, and 7.1.6 for respectively *S.* Enteritidis load in caecum content, liver and spleen. Overall the phenotypic variation explained by each gene is small, most of the phenotypic variation varies between 4% and 8% for each gene for *S.* Enteritidis load in the three organs.

## DISCUSSION

In this study, associations of 12 candidate genes were investigated with *S.* Enteritidis load in caecum, liver and spleen after an experimental infection of three-week-old meat-type chickens. Even though not all polymorphisms examined resulted in an amino acid substitution, significant associations or tendencies were found for all candidate genes. This might be due to linkage to functional polymorphisms in same or nearby genes. All of the gene SNP tests were developed at Iowa State University, using Iowa resource populations, containing broilers, leghorns and Fayoumi lines, and with this study confirmed in the Dutch population. Since these populations are unrelated, these results validate the association of these candidate genes with *S.* Enteritidis response. The results also suggest that there is not a lot of variation in these genomic regions between different populations.

*NRAMP1* is a membrane transport protein, which regulates intracellular growth of various pathogens (Hu et al. 1996). *NRAMP1* showed a significant association with bacterial load in caecum in the current study, and there tended to be an association with bacterial load in spleen and liver. The same polymorphism was previously described by Liu et al. (2002), and they report a significant association of *NRAMP1* with *Salmonella* load in spleen in young chicks. Because the SNP is silent, it may be linked to causal mutations that differ in allelic association between populations. Three other genes are also involved in the pathogenesis of *Salmonella*. *IAP-1* and Caspase-1 are involved in the apoptotic pathway when intracellular bacteria interact with host cells (Deveraux and Reed 1999; You et al. 1997) and *PSAP* was selected as a positional candidate (Kaiser et al. 2002; Schmid et al. 2000). *PSAP* generates saposins that are activators of lysosomal enzymes

(Madar-Shapiro et al. 1999). Lamont and co-workers (Lamont et al. 2002; Liu and Lamont, in preparation) also studied the genes *IAP-1*, Caspase-1 and *PSAP*, and showed an association with both caecum and spleen bacterial load with Caspase-1, and an association with spleen bacterial load with *IAP-1* and *PSAP*. In the current study, significant associations with *S.* Enteritidis load were found of *IAP-1* and *PSAP* in caecum and of Caspase-1 in caecum and liver.

The gene *iNOS* is involved in the L-arginine pathway that produces, for example, nitric oxide (NO). NO is mainly produced by activated phagocytes and acts as a cytotoxic agent (Schmidt and Walter 1994). Macrophages from chickens of different genetic background differ in *iNOS* activity, expression and regulation (Hussain and Qureshi 1997; 1998). The significant effect of *iNOS* on *S.* Enteritidis load in caecum might reflect the importance of this gene in genetic resistance to *S.* Enteritidis. Another interesting finding in the current study was a significant interaction between *iNOS* and *NRAMP* in-group 3 for *S.* Enteritidis load in caecum ($P = 0.03$). Both of these genes are important factors produced by macrophages that might interact at a molecular level.

Two cytokines, interleukin-2 (*IL-2*) and interferon gamma (*IFNγ*) were investigated. *IL-2* induces the proliferation and differentiation of T, B and NK cells (Janeway and Travers 1997) and *IFNγ* increases the expression of MHC Class I and Class II molecules that modulates the immune response (Janeway and Travers 1997). These two cytokines were described in an association study with antibody response kinetics (Zhou et al. 2001a). It is likely that the cytokines, *IL-2*, which induces growth of T-, B- and NK-cells, and *IFNγ*, which is the main macrophage activating cytokine, might play an important role in the *Salmonella* load in the various organs. This was confirmed with the association found for *IL-2* with *S.* Enteritidis load in both caecum and liver, moreover there tended to be an association between the *S.* Enteritidis counts in spleen and caecum and *IFNγ*.

*IgL* and *ZOV3* are two genes that are members of the immunoglobulin superfamily (Kunita et al. 1997; Zhou et al. 2001a; Zhou et al 2001b) *IgL* and *ZOV3* are important in, for example, interactions with antigen-presenting cells. In this study, both *IgL* and *ZOV3* were associated with *S.* Enteritidis load in caecum and liver. In a previous study, these genes were significantly associated with antibody response kinetics (Zhou et al. 2001a). A family of genes that are essential in all kinds of basic biological processes are *TGFβ* genes (for review see Newfeld et al. 1999; Massague and Chen, 2000). In this study, significant associations of the various *TGFβ* genes were found with caecum, liver and spleen *S.* Enteritidis load. Significant associations of these genes on growth and composition and antibody kinetics were previously described in Iowa resource populations (Li et al. 2002; H. Zhou, personal communication). In the current study, no significant interactions were found between these genes and one of the traits according to *S.* Enteritidis load measured. Thus, besides the effect of these genes on growth and composition (Li et al. 2002) and

antibody kinetics (H. Zhou, personal communication), these genes might effect the *Salmonella* load in various organs.

Comparing *S.* Enteritidis load in the various organs between the three genotypes of the different genes, sometimes the heterozygous genotype had the lowest *S.* Enteritidis counts, not one of the homozygous genotypes. To keep genetic diversity and variation in a population, it is favorable to keep as many heterozygous animals as possible. Moreover, it is a general rule that high levels of genetic variability are associated to evolutionary health (Amos et al. 2002). Heterozygosity in genes affecting immunity might allow a wide range of protective responses.

After calculating the phenotypic variation explained by each gene, it appeared that the effect of each gene in *Salmonella* resistance is small. Most of the genes explained 4% to 8% of the phenotypic variation. This could also be expected, since *Salmonella* resistance is known to be a polygenic trait, tendencies or significant associations were found for all genes investigated in the current study. Resistance to *Salmonella* depends on many factors, the invasion of *Salmonella*, the adherence of *Salmonella* to cells, and the activity of the immune system, and most of these factors are also depended of age. Thus, most likely, there are a lot of factors that can influence the resistance of a chicken to *Salmonella*. For genetic selection of *Salmonella* resistance, multiple genes should be selected to enhance the effect and to receive the optimum result.

The investigated candidate genes play an important role in part of the immune system important after *Salmonella* infection, and most of these genes showed an association with *Salmonella* counts in one of the organs. Overall, most significant associations were found between candidate genes and caecum *S.* Enteritidis load. This was unexpected, because spleen is an important immunological organ, and thus we expected more associations with spleen bacterial counts. However, the caecum also plays an important role in the dissemination of *Salmonella* throughout the body of a chicken, and it provides a sampling of the bacterial load in the digestive tract, which is responsible for horizontal transmission of *Salmonella*. Thus these results suggest that is possible to select for genes that result in lower *Salmonella* count in caecum. This might decrease the development of a systemic infection and the spread of *Salmonella* throughout the flock. The findings of the current study make the investigated genes informative and important in genetic resistance to *Salmonella*, and thus interesting to use for breeding strategies. However, further research needs to be done to confirm that these genes are actually causative genes.

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# Chapter 7.2

## DIFFERENTIAL GENE EXPRESSION IN CELL LINES AFTER *SALMONELLA* INOCULATION

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**SUMMARY**

The modified differential display technique was used to investigate differences in gene expression between macrophages inoculated with *Salmonella* Enteritidis and non-inoculated macrophages during a time course from 0 to 48 hours post inoculation. In a brief investigation differences in expression profiles during time were found for three specific transcripts. These results indicate that it is possible in further studies to identify genes involved in particular traits like resistance to *S.* Enteritidis based on the transcriptional behavior. Furthermore it is possible to discriminate between early, middle and late responsive genes. With this experiment differential display proved to be successful to find new potential candidate genes.

## INTRODUCTION

Studies showed that considerable genetic variation exists between and within broiler lines with respect to susceptibility to *Salmonella* (Bumstead and Barrow, 1993; Beaumont et al., 1999; Kramer et al., 2001). Furthermore, these studies showed that disease resistance to *Salmonella* is polygenic. Chapter 7.1 described the association of several genes with *Salmonella* colonization in selected organs. In order to identify new, additional, genes that might influence *Salmonella* resistance, analysis of differences in RNA expression levels between animals or cell lines can potentially be of value.

Differential display is a powerful approach that enables the identification of genes whose RNA transcripts are differentially expressed between samples. To identify genes involved in *Salmonella* resistance in chicken, the differential display reverse transcriptase polymerase chain reaction (DDRT-PCR) technique was optimized. This technique was originally described by Liang and Pardee (1992). The DDRT-PCR technique was performed essentially as described by Kohroki and coworkers (1999) but with several modifications that relate to work by others (Wang and Rowley, 1998; Ivashuta et al., 1999; Zhang et al., 1999; Wang et al., 2000). First of all, each gene is represented by only one fragment, which is the 3' end of the mRNA transcript. Secondly, adapters were ligated to the 5' end of these fragments to facilitate more stringent amplification conditions. Thirdly, amplification was performed by fluorescently labeled primers allowing for more fragments to be analyzed in a single run due to different fluorescent labels in combination with anchored primers. Fragments were electrophoresed on a poly-acryl-amide gel to allow automatic analysis of the results.

Here the DDRT-PCR technique is applied to identify novel genes involved in resistance to *Salmonella*. In this chapter, the technique was validated using a chicken macrophage cell line, inoculated with *Salmonella*.

## MATERIALS AND METHODS

*Cell lines and Salmonella inoculation*

The chicken macrophage cell line HD11 was inoculated with *Salmonella* Enteritidis (multiplicity of infection (MOI) = 10) as described in chapter 2 (Kramer et al., 2003). Thirty minutes after incubation, extracellular *S.* Enteritidis were killed by adding gentamycin (Kramer et al., 2003) and subsequently cells were harvested for RNA isolation 0.5, 1, 4, 12, 24 and 48 hours post *S.* Enteritidis inoculation.

*Differential Display Technique*

The procedure of differential display is schematically presented in Figure 7.2.1.

RNA isolation

Total RNA was isolated with the RNeasy kit (Qiagen, Westburg, Leusden, The Netherlands) following the manufacturer's instructions. An on-column DNaseI treatment was included in the protocol. The isolated total RNA was tested for integrity by 1% agarose gel electrophoresis and quantified spectrophotometically at 260 nm.

cDNA synthesis

First and second strand cDNA synthesis (step 1 and 2 of Figure 7.2.1) was performed according to the Superscript II RNase H deficient reverse transcriptase protocol (Life technologies FocusOn technical bulletin 18064-3) using an equal mixture of five 3' anchored and 5' biotinylated oligo-dT primers (5' biotin-TTTCGGAATTCCGTCGACCGT14-A/G/CC/CA/CG) in the first strand cDNA synthesis reaction in stead of standard oligo-dT primers. The biotin modification was included for 3' cDNA fragment recovery as discussed later. The anchored nucleotides were introduced to exclude poly-dA sequences in the resulting cDNA templates as described by Wang and coworkers (2000). An EcoRI site was included in the primer sequence to remove the cDNA fragments from the biotin-streptavidin-beads complex.

Adapter ligation

Biotinylated double stranded cDNA was bound to streptavidin coated magnetic beads (Genovision, Oslo, Norway; step 3 of Figure 7.2.1) according to the manufacturer's protocol. The cDNA-bead complex was washed and resuspended in water. Subsequently, the cDNA-bead complex was simultaneously digested with *HhaI* (New England Biolabs, Westburg, Leusden, The Netherlands) and ligated to HhaI adapters (5' GACGATGAGTCCTGATCG and 5' ATCAGGACTCATCG; step 4 of Figure 7.2.1).

The adapter-ligated biotinylated 3' cDNA bead complexes were separated from all other adapter ligated cDNA fragments by magnetic separation, and after washing resuspended in water.

3' cDNA recovery

The adapter ligated 3' cDNA fragments were removed from the biotin-magnetic bead complex by restriction digestion with 20 units *EcoRI* (New England Biolabs) in a total volume of 100 µl for 4 hours at 37ºC (step 5 of Figure 7.2.1). Subsequently, the magnetic beads were removed from the reaction by magnetic separation. The resulting solution containing adapter-ligated 3' cDNA fragments was ready to use as a template for amplification.

PCR amplification

The template 3' cDNA fragments were amplified by a semi quantitative polymerase chain reaction (step 6 of Figure 7.2.1) in a reaction volume of 20 µl containing 2 µl of template 3' cDNA fragments, 1 × PCR buffer, 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.25 µM of each primer and 1 unit of AmpliTaq polymerase (Perkin Elmer). The primers used were a combination of either of 11 fluorescently (FAM, HEX or TET) labeled anchored primers (5' CCGTCGACCGTTTTTTTTTTTTTTAA/AT/AG/AC/GA/GT/GC/GG/CA/CG/CC) and either of four HhaI adapter specific primers (5' GATGAGTCCTGATCGCA/G/C/T). This leads to 88 different combinations of primers that each amplifies a subset of the 3' cDNA fragments.

The PCR cycling profile was 72ºC for 2 minutes (adapter elongation), 95ºC for 8 minutes (initial denaturation), [92ºC for 20 seconds, 56ºC for 30 seconds, 72ºC for 40 seconds] for 24 cycles followed by 72ºC for 8 minutes (final elongation).

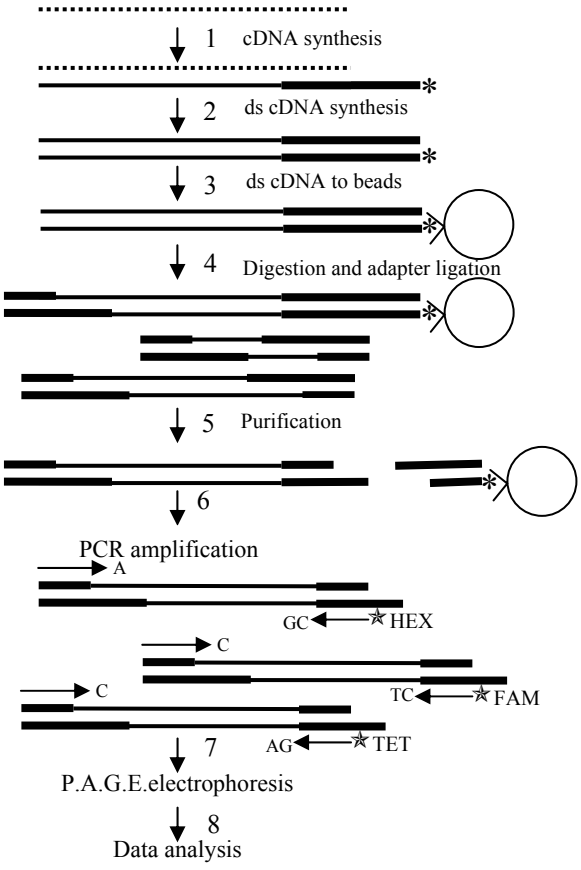


**Figure 7.2.1.** The differential display procedure.

1. cDNA synthesis using a biotinylated primer
2. ds cDNA synthesis
3. ds cDNA linked to beads using biotinylated primer
4. digestion of ds cDNA with *HhaI* and adapter ligation to restriction sites
5. purification of ds cDNA from beads by *EcoRI* digestion
6. quantitative PCR amplification of fragments
7. PCR fragments were sized by PAGE and data was digitally collected
8. Automatic analysis of the data for the abundancy of each transcript

Differential display analysis

PCR products were mixed with fluorescently (TAMRA) labeled marker (Genescan 2500; Applied Biosystems), denatured and loaded on a 8% poly-acryl-amide sequencing gel and run for 14 hours in a ABI 373 sequencer (Applied Biosystems). Fluorescent data was collected and analyzed by Genescan and Genotyper 1.1.1 software (step 7 and 8 of Figure 7.2.1). Each peak represents one or several specific 3' cDNA fragments of a particular size and the peak height was taken as the relative abundance of the corresponding mRNA transcripts. Peaks were visually checked for differentially amplified peaks.

## RESULTS

Results of automatic fragment analysis are presented in Figure 7.2.2. This figure shows the differences in expression profiles between non-inoculated and inoculated cells of fragments in the range of 290-330 bp of a single primer combination during a time-course experiment. The fragment of 322/323 bp was not differentially expressed, since the height (and the total surface) of these peaks do not differ much between samples, i.e. the ratio between the height of the non-inoculated cells and the inoculated cells was close to one (Table 7.2.1). The other two identified fragments of 297 and 307 bp were differentially expressed in time from 12 hours on. At 4 hours post inoculation no differences in expression levels were found for these fragments. The fragment of 297 bp was more abundantly expressed in infected cells 12 hours post inoculation (more than two times as much, Table 7.2.1), and even more at 24 hours post inoculation (almost four times as much, Table 7.2.1) as compared to the non-inoculated cells. The expression fragment of 307 bp was most abundantly present at 12 hours post inoculation compared to the non-inoculated cells (2.5 times as much Table 7.2.1), and declined onwards. Both differentially expressed fragments returned to expression levels comparable to that of non-inoculated cells at 48 hours post infection. This means that genes represented by these fragments of 297 and 307 bp are differentially expressed during the time-course of the *Salmonella* challenge.

**Table 7.2.1.** Peak height ratios of three RNA transcripts during the time-course of *Salmonella* inoculation relative to non-inoculated cells

| Hours post inoculation | Fragment length | | |
|---|---|---|---|
| | 297 | 307 | 323 |
| 4 | 0.9 | 0.9 | 1.0 |
| 12 | 2.3 | 2.5 | 1.0 |
| 24 | 3.8 | 1.5 | 1.0 |
| 48 | 1.0 | 0.7 | 0.9 |

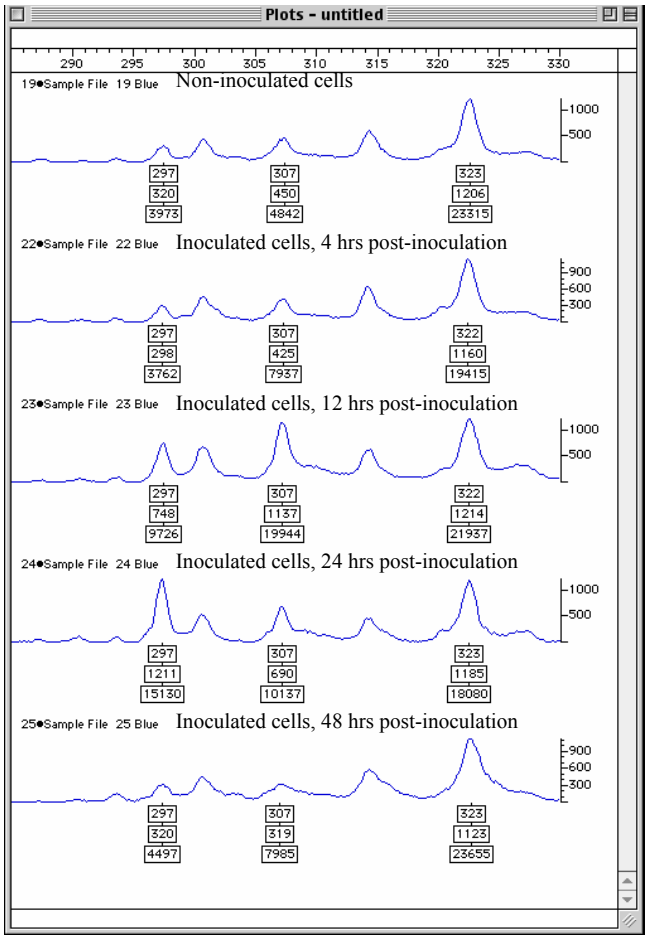


**Figure 7.2.2.** Automated gel analysis of RNA profiles of non-infected cells, and infected cells at 4, 12, 24 and 48 hours post infection. Three peaks are identified in each sample, each peak is labeled with the size of the band (upper number), the height of the peak (second number) and the total surface beneath the peak (third number). The first two labeled fragments (297 and 307 bp) in each profile are differentially expressed, and the third labeled fragment (of 322/323 bp) is not differentially expressed.

## DISCUSSION

The present study reports a brief investigation into the potential of transcriptional analysis after *Salmonella* inoculation of a chicken macrophage cell line Therefore, we applied the modified DDRT-PCR technique. Two differentially expressed genes were found after a macrophage cell line was inoculated with *Salmonella* (Figure 7.2.2). Moreover, these fragments were differentially expressed in time. However, after the current procedure (Figure 7.2.1.), it seemed to be difficult to isolate the specific fragments to identify the genes represented by these fragments. So, the interesting fragments could not be identified.

Results prove that it is possible with the DDRT-PCR technique to identify differentially expressed genes, and that it is possible to discriminate between early, middle and late responsive genes. These results are promising for further research to identify differences in expression profiles of *Salmonella* susceptibility genes in cell lines in time. It should also be possible to find differences in expression levels between cells or macrophages from tissues of susceptible and resistant chicken, using the differential technique. When several genes are identified that are involved in differences in genetic resistance between animals, it is possible to identify pathways involved in resistance to *Salmonella*.

In the near future it will be possible to use micro arrays to identify differences in gene expression between samples. With this technique it is possible to screen much more genes at the same time, leading to specific new genes and pathways. Changes in macrophage gene expression induced after *Salmonella* infection or *Salmonella* lipopolysaccharide stimulation has been previously described in mice using micro arrays (Rosenberger et al., 2000). These results provide insight into host-pathogen interaction, how intracellular bacteria use some of their virulence effectors to specifically alter host cell biology and secure their niche.

After identifying genes and pathways involved in disease resistance to *Salmonella*, these genes can be further investigated for their effects. Differences in expression profiles are difficult to use when animals need to be selected for breeding activities, because it is time and labor consuming to type each animal. Thus other assays need to be developed for high throughput screening of animals. Therefore, genomic DNA sequences of those interesting genes can be used to find single nucleotide polymorphisms (SNP) in the genome, like described in chapter 7.1. The effect of these SNPs on the trait of interest, *Salmonella* resistance can be analyzed. This will eventually lead to low cost tests to improve genetic resistance of chickens to *Salmonella* and other pathogens.

# Chapter 8

## GENERAL DISCUSSION

Infectious diseases and zoonoses cause animal disease, economic losses and human disease, and are therefore still a major problem in the poultry industry. In the 1940's commercial poultry breeders spent considerable effort on selection for resistance against infectious diseases, but when vaccines and antibiotics became available, the selection effort was commonly received as unnecessary, and it was widely abandoned (Knap and Bishop, 1999). So, for the last fifty years the poultry production sector has mainly relied on vaccination, medication, and management techniques for the control of animal health and zoonotic agents, and as a consequence health related traits have played a minor role in animal breeding. However in the last decade, there is a growing concern in the western society over excessive use of medications like antibiotics and vaccines. Moreover, the costs of treatment and veterinary care increases, sometimes even faster than the value of the animals. Therefore, breeding for genetic resistance to infectious diseases again becomes more attractive. Enhanced resistance to disease is stable under natural selection, and therefore artificial selection for disease resistance should also be stable and sustainable (Stear et al., 2001). Moreover, enhanced disease resistance will result in reduced transmission of the disease from one host to the entire flock, or from flock to flock (Bishop and Stear, 1997). Not all animals of a flock need to be resistant, the main objective is the reduction of the transmission of the infection among individuals, in order for the infectious agent to fade out. It is important that not all animals need to be resistant, because under those conditions it is possible to keep genetic diversity in the flock, which might be desirable for future breeding strategies.

In this thesis, genetic resistance to *Salmonella* was measured by quantifying bacterial load in liver and spleen as a measure of systemic infection, and bacterial load in caecum content as a measure of colonization in the gut and shedding. To lower the transmission rate, it is important to reduce the shedding of disease causing agents by chickens or to enlarge the threshold, by which an individual becomes infected. Ultimately, with this strategy it is possible to eradicate the infectious agent from the flock. It is also important to decrease the bacterial load in systemic organs. Young animals can be highly susceptible to infectious diseases when the infection becomes systemic, and such an infection can be lethal to chicks. Another important aspect to diminish the systemic infections is that when birds are immunocompromised by *e.g.* stress during transport to the slaughterhouse, an enormous increase in bacterial load in caecum can occur. This increase might be due to bacteria present in systemic organs or caecum, because these bacteria are able to multiply very fast when the host is immunocompromised. With this strategy, selection on reduced shedding and colonization of the bacteria in the host, it should be possible to breed animals resistant to *Salmonella* at various ages. However, with these selection criteria the effect of life vaccines orally administered, might be reduced, since the

bird might not only be more resistant to colonization of pathogenic strains but also to the colonization of life vaccines.

Breeding for disease resistance is a challenging approach. The progress per generation might be small and costly, but it is heritable and therefore cumulative over generations. Occasionally a single host gene determines the host's susceptibility to infectious disease, *e.g.* the presence of the F4 receptor in the small intestine completely protects the pigs against a challenge infection to Enterotoxigenic *Escherichia* Coli serotypes with adhesin F4 (Cox et al., 2002). More frequently, however, several host genes interact with each other in a complex way, with each gene contributing to a different extent for susceptibility or resistance to infection. So, a lot of factors can influence the success of breeding for disease resistance. Besides the genotype of an animal that influences resistance, animals should be healthy, as stress, other diseases or non-hygienic circumstances make an animal much more susceptible to infections. Moreover, when breeding for resistance for a specific pathogen, it is desired to include also other bacteria in these selection criteria. In the current thesis the importance of the innate immune response and the intracellular survival of *S.* Enteritidis was investigated. With this strategy, to investigate disease resistance to bacteria like *S.* Enteritidis, it might be possible to select generally for resistance to other intracellular bacteria, like other *Salmonella* serotypes like *S.* Typhimurium, or other bacteria like Listeria. The existence of a more general mechanism of resistance to all *Salmonella* serotypes was previously indicated by Bumstead and Barrow (1993). However, it is complex to breed animals resistant for multiple bacterial, viral and parasitic infections, the pathogenesis and reaction of the host to various infections caused by diverse pathogens can differ enormously.

Next to adherence and invasion, immunoresponsiveness is a major factor determining resistance to disease. Two types of immune responses, the innate and adaptive immune response, form a complex interacting system together with numerous other biological processes to provide a good response after an infection in chicken. Therefore, in this thesis we tried to characterize the immune response against *Salmonella* in chickens in more detail (chapter 2, 3 and 4), before actually trying to identify markers or genes for *Salmonella* resistance (chapter 5, 6 and 7).

### *SALMONELLA* RESISTANCE AND THE IMMUNE SYSTEM

After a chicken becomes infected, the immune response will be activated. The first line of defense is the innate immune response, where at first phagocytes (like macrophages or heterophils) will start moving to the site of infection to start to eradicate the infection and organize the local immune response. However, *Salmonella* is a bacterium that has the ability to enter and survive in all kinds of immunological cells, like for example phagocytes

(macrophages) or lymphocytes. From these cells, macrophages appeared to be the most effective cells in clearing *Salmonella* that entered the cells (chapter 2). Because *Salmonella* is able to survive within cells, it is possible that these cells (*e.g.* phagocytes), which can be transported throughout the body, cause a systemic infection by infecting other organs. Another effect of the ability of *Salmonella* to survive within cells might be that *Salmonella* can not be detected in the intestine, but during periods of stress or intercurrent infections recrudescence can occur from internal reservoirs (like spleen, liver, or caecum). In a short period of time, these carrier chickens can than have high numbers of bacteria in their gut (Qin et al., 1995; Desmidt et al., 1997). So, the innate immune response (in particular the phagocytes) is important in a *Salmonella* infection. The innate immune response not only plays an important role in preventing an infection and clearance after an infection by *Salmonella* in chicken (as also demonstrated in chapter 3), but it might also play an important role in the dissemination of the bacteria throughout the body of the chicken. Thus, traits like the innate immune response, and in particular the phagocytes, may be of major importance when breeding for genetic resistance to *Salmonella*. Moreover, because the innate immune response is nonspecific, if genes or markers are found that play an important role in resistance to *S.* Enteritidis, those factors may also be important in resistance to other intracellular bacteria.

To identify whether their might be general factors involved in disease resistance, it was important to describe differences in natural resistance for genetic groups of chickens (chapter 4). These genetic groups showed significant differences between several immune parameters that were measured in the absence of an infection. Actual differences in resistance to *Salmonella* challenge between genetic groups (as described in chapter 5 and 6) could be linked to the importance of different parts of the immune system. Particularly the innate immune response played an important role (chapter 4, 5 and 6) as was expected considering results form chapter 2 and 3. Overall, this research described the differences in immune responses between and within groups, and the importance of the innate immune response in genetic resistance to *Salmonella*.

In mice it was indicated that the innate capacity to mount an acute inflammatory response to *Salmonella* might be beneficial for host resistance to intracellular bacteria (Araujo et al., 1998). Moreover, Fierer (2001) demonstrated the important role of the innate immune system, in particular polymorphonuclear leukocytes, in resistance to *Salmonella* in mice. In chicken, Kogut and coworkers (Kogut et al., 1994; Stabler et al., 1994) demonstrated the relation between the innate immune system and resistance to *Salmonella*. They showed that, *in vitro*, heterophils could more efficiently internalize and kill *Salmonella* compared to the avian monocyte. The important role of the innate immune response was also indicated in a study from Bumstead and Barrow (1993), who suggested that there might be a general mechanism of resistance to various *Salmonella* serotypes. Moreover there

seemed to be a relationship between the age of chicken, the functional activity of the heterophil, and the susceptibility to organ invasion by *Salmonella* (Wells et al., 1998). Usually, birds become infected by *Salmonella* at a young age when they are most susceptible (chapter 5), thus it might be favorable for resistance when it is possible to increase the activity of the innate immune system the first days post hatch by selection. They also suggested that IL-8 is a major chemotactic factor produced by the host, which aids in mediating the *Salmonella*-induced recruitment of heterophils to the site of *Salmonella* invasion (Kogut, 2002). Qureshi and coworkers performed research on the function of the avian macrophage and the interaction with pathogens. They indicated that genetic lines differ in peritoneal macrophage function in response to *Salmonella* and other pathogens (Qureshi et al., 1986; for review see Qureshi et al., 2000). These studies are in agreement with the results presented in this thesis on the role of phagocytes in disease resistance to *Salmonella*.

However, although the innate immune response plays a major role in the first line of defense in infectious diseases, the adaptive immune response is also of great importance in the clearance of *Salmonella*. Arnold and Holt (1995) showed that the immunocompetence of a bird relied on interdependent functions of multiple components of the immune response, *i.e.* aspects of both innate and adaptive immunity. They demonstrated that the intestinal shed rate of *Salmonella* increased after treatment with immunosuppressive drugs for humoral immunity. This showed that the elimination of *Salmonella* also depends on humoral immunity, which was also indicated by Desmidt et al (Desmidt et al., 1998). Although the T-cell responses after a *Salmonella* infection in chicken was not investigated thoroughly, our results indicated that next to the innate and humoral immune response, T-cell proliferation is of importance after an infection. So for the first response a chicken is dependent on the innate immune system, which seemed to be a major factor in disease resistance, but also adaptive immune responses play a role in subsequent clearing of bacteria from the host. Results described in chapter 3, 4 and 5 of this thesis, also confirmed the raise of the adaptive immune response later on in the infection that might influence resistance to *Salmonella*.

Other studies already described the polygenic nature of disease resistance to *Salmonella* in poultry (*e.g.* Bumstead and Barrow, 1993; Lindell et al., 1994; Protais et al., 1996; Cotter et al., 1998; Girard Santosuosso et al., 1998; Kaiser and Lamont, 2001). Differences in genetic resistance could be associated with several candidate genes (Lamont et al., 2002; chapter 7.1). Different candidate genes are involved in the immune response that arises after a *Salmonella* infection. Not only *in vitro* assays showed a link between genetic resistance to *Salmonella* and the innate immune system, also some candidate genes involved in innate immunity showed a significant association in response to a *Salmonella* infection. Before using molecular markers for disease resistance, it is important to define selection criteria, because markers might differ if birds need to be selected on the

level of systemic infection, bacterial counts in caecum, or carriers. Differences in RNA expression levels can also be a strategy to search for more candidate genes involved in *Salmonella* resistance. With this method differences in expression levels of early, middle, and late responsive genes involved in host–pathogen interactions could be characterized using cell lines infected with *Salmonella* (as described in chapter 7.2). These genes might play a role after an *in vivo* infection. It is also possible to compare animals that are more resistant with animals that are more susceptible to infections, which might lead to other genes involved in the pathogenesis.

**IMPLEMENTATIONS**

Genetic improvement through artificial selection has been an important contributor to the enormous advances in productivity that have been achieved over the past 50 years in plant and animal species that are of importance for human food production. Most of these traits are controlled by several genes, and have quantitative effects on the genotype. So far most selection has been on observable phenotype (Dekkers and Hospital, 2002). Two important strategies for genetic improvement are recurrent selection and introgression programs. In livestock, recurrent selection is the main vehicle for genetic improvement, the aim is to improve a breed or a line as a source of superior germplasm for commercial production through within-breed or within-line selection. For example the progeny test, in which breeding values are estimated on the basis of the phenotype of progeny that have been created through test matings. The other strategy, introgression, is mainly used in plants, the aim is to introduce a target gene from a donor breed into a productive line that lacks that particular gene. This strategy often requires more generations to obtain sufficient individuals for further breeding or if more than one gene must be introgressed. This makes it unfavorable for breeding in livestock (Tanksley et al., 1989; Dekkers and Hospital, 2002).

The use of genetic markers in genetic improvement programs can be useful for traits like disease resistance. Because often the heritability of phenotypic traits for disease resistance are low, and the difficulties and expenses of recording phenotype that might be necessary can be avoided when using molecular markers. For a single marker, the molecular score of an individual is obtained as the estimate of the statistical association between marker genotype and phenotype. If multiple markers are involved, genotype effects can be summed over all markers into a single molecular score (Lande and Thompson, 1990). Selection on a combination of molecular score and the phenotypic information is the most powerful strategy (Dekkers and Hospital, 2002). This genetic improvement can be achieved by using recurrent selection programs of disease resistance to calculate breeding values for the parents, using genotypes or phenotypes of relatives. These breeding values will be used for the next generation. Introgression can also be used for improving disease resistance using

molecular markers. If a breed or line is more resistant to a disease (donor) than another more susceptible breed or line (recipient), than molecular markers can be used to introduce the target gene from the donor into recipient by crossing these two breeds. However, as mentioned before, this requires more generations to obtain sufficient individuals for breeding livestock than recurrent selection. In case of disease resistance to *Salmonella* in poultry, most likely the recurrent selection program within one breed or line is favorable, since it requires less generations to introduce a polygenic trait like *Salmonella* resistance in a flock using several molecular markers and phenotypic information.

Although these selection strategies are very powerful, and the speed of selection can be enhanced because it is possible to select animals on early age, it is important to make sure that there is no association with other, undesired traits together with the target trait. For example in disease resistance, it is most likely that the favorable target genes are involved in the immune system. Since the immune system is a complex system that is involved in many pathways, it should be thoroughly investigated whether production traits or other traits are not negatively influenced by selection on disease resistance. Moreover various mechanisms of host immunity may not be intercorrelated, or possibly unfavorable intercorrelated (Rothschild, 1991; Mallard et al., 1998). Additionally, an increase of "general" immunocompetence does not necessarily lead to an increase in resistance to a specific disease (Biozzi et al., 1982; Knap and Bishop, 1999). However, even if unfavorable associations exist, selection indices can be created that includes traits with unfavorable associations and maximize the desired responses while attempting to minimize undesirable effects (Stear et al., 2001).

It is also important to select animals for disease resistance in their natural environment. Under other circumstances and surroundings than their natural habitat, animals might respond in a different way to diseases. Moreover, it is important to mimic an experimental infection comparable with the natural route of infection. This might influence results of experiments if not conducted in an accurate way, through which it is possible that in breeding programs animals are selected with the wrong criteria.

The advantage of studying disease resistance as described in this thesis is the use of one specific, well defined pathogen *S.* Enteritidis PT 4. Since only one pathogen is used, there is no interference with other pathogens that might influence the results. Moreover, *S.* Enteritidis PT 4 is a well known and described, often used pathogen, which makes it easy to work with. However, an important aspect of breeding for disease resistance in practice might be the fact that the animal is more resistant to multiple different pathogens. Nevertheless, to elucidate all factors involved in selecting for disease resistance at the molecular level, we used only one pathogen in the current study as described in this thesis. This provides a basis for further experiments to measure differences in disease resistance to multiple pathogens.

Developments in human- and animal genetics (like this thesis) enable the rapid identification of genes that influence the susceptibility to, or the course of, infectious disease. Most of these genes are involved in adherence, invasion and colonization of microorganisms, innate and adaptive immunity, and inflammation. These mechanisms are mostly equivalent between human and animals thus a lot of knowledge about breeding disease resistant animals, can be used in human research. Knowledge of the genetics of infectious diseases clarifies the molecular basis of susceptibility or resistance, may refine therapy, and may lead to the identification of novel targets for the development of new drugs and vaccines (Kimman, 2001). However, the objective in animal research is to decrease bacterial load in organs and to reduce shedding by the animal (and subsequent transmission), whereas with human research it is important to prevent clinical disease. Thus research must be emphasized to specific criteria. Different markers can be found when searching for markers that are related to systemic infection, carriers, shedding or maybe clinical disease. Overall, not only can this type of research be used for animal breeding, it also has its prospects in infectious diseases in human.

Results presented in this thesis showed that it is possible to breed animals for *Salmonella* resistance. Therefore, differences in immune response can be used as selection criteria as described in chapter 2 to 6. However, candidate genes are more convenient to characterize animals as described in chapter 7. Overall, this study confirmed that disease resistance to *Salmonella* is a polygenic trait, which makes it more difficult to apply in breeding strategies. Most likely it is too complex to breed animals that are completely resistant to *Salmonella* within the next few years. Breeding for disease resistance might result in a lower *Salmonella* infection level of the birds and a higher threshold to become infected, and hence reduced transmission of the infection throughout the flock. However, it is important to confirm that no other undesired production traits are selected together with the target trait. To conclude, together with other existing strategies to control *Salmonella*, the genetic approach may provide additional means to reduce *Salmonella* contamination in poultry for safer human food products.

# SUMMARY

*Salmonella*, a gram-negative facultative intracellular bacterium, is a worldwide problem as a human disease and as an economical problem for the poultry industry. Salmonellosis is one of the most common food-borne diseases in humans, human infections cause gastroenteritis, which may result in death in highly susceptible individuals, like the elderly or young children. Salmonella also causes economical problems for poultry farmers, since Salmonella might lead to high levels of mortality of a part of the flock, and decreased animal welfare. *Salmonella* Enteritidis has the capacity to produce severe illness and mortality in young chickens, while adult birds can be infected with *S.* Enteritidis without any symptoms of disease. After a chicken is infected, the entire flock can easily become infected as a consequence of horizontal transmission. To prevent the consequences of these infections, birds can be vaccinated or treated with antibiotics. However, over the last decade, the use of antibiotics has been criticized because of the possible development of antibiotic resistant organisms and the potential dangers of residual antibiotics in the human food chain. Another possible way to eradicate disease in poultry is by management strategies, such as different type of feed, housing or better hygiene, but this is not always possible and is quite costly. A third strategy to prevent or control an infection is breeding birds that are genetically more resistant to *S.* Enteritidis. This can be exploited by improving the immune responses, so that the possibility for residues of antibiotics in food products can be reduced. Moreover, the speed of selection can be enhanced because it is possible to select animals at early age.

The natural route of an infection with *Salmonella* usually starts via the oral route followed by colonization in the gut. Then *Salmonella* is able to penetrate the mucosal epithelium of the small intestine, which triggers the innate immune response by chemotaxis of phagocytic cells to the site of infection. These phagocytic cells start to eliminate the pathogen, and activate the adaptive immune response (for more details, see **chapter 1**). To obtain an early clearance of the pathogen after an infection, the first line of defense, the innate immune response, appears to be essential. An early clearance of *Salmonella* is important when breeding for genetic resistance to *Salmonella*. Therefore, this thesis mainly focussed on the role of innate immune response after a *Salmonella* infection.

The objective of this thesis was to develop markers for phagocyte functions and their role in resistance to *S.* Enteritidis infection in broilers. Therefore, *in vitro* and *in vivo* experiments were carried out to characterize the role of various parts of the immune system, especially of the innate but also of the adaptive immune responses. The importance of selected cells of the innate immune response, the macrophages, after an *in vitro* infection is demonstrated in **chapter 2**. This chapter describes the entry and survival of *S.* Enteritidis in chicken macrophage and lymphocyte cell lines (both T- and B-cells). Although *S.* Enteritidis was able to survive in all tested cell lines (macrophages and lymphocytes), results showed that macrophage cell lines were able to internalize high numbers of bacteria, and thereafter,

effectively start clearing the intracellular bacteria. Lower numbers of bacteria entered the lymphocyte cell lines than in the macrophage cell lines, and lymphocytes were not able to start clearing the infection as fast and effective as the macrophage cell lines. Thus macrophage cell lines seemed to be more effective in clearing an infection than the lymphocyte cell lines. The importance of the innate immune system after an *in vivo* *S.* Enteritidis infection is described in **chapter 3**, where White Leghorn chickens were infected with *S.* Enteritidis. Because the innate immune response seemed to be important short after an infection, chickens were sacrificed at 0, 24, 48 and 72 hours post infection, and various innate immune parameters were measured. These results showed that the function of phagocytic cells increased between 0 and 72 hours post infection, due to either higher numbers of phagocytic cells in the organs or due to increased activity. Results described in these two chapters underline the important role of phagocytes after an infection.

Previous to the research of differences in resistance after a *S.* Enteritidis infection, it is important to measure the differences in natural resistance on a fundamental physiological level (**chapter 4**). The differences in natural resistance between seven genetic groups of chicken were determined by measuring differences in immunocompetence. Therefore, the natural immune responses were characterized of chickens of seven groups form 2 to 5 weeks of age. Results showed that there are considerable differences in immunocompetence between different genetic groups for immune responses that are important in a chicken after a possible *Salmonella* infection. All groups of chickens were able to give rise to a proper immune response, using either the innate or adaptive immune response, although none of the groups was uniformly superior with respect to all traits measured. Thus, for reliable measurements of general immunocompetence or resistance to *S.* Enteritidis it is important to investigate several parts of the immune system. Further research was conducted to measure whether the differences in immune profiles and natural resistance could be linked to actual disease resistance.

The differences in innate and adaptive immunity between four different groups of chickens were identified after an oral infection with *S.* Enteritidis (**chapter 5**). The level of infection was measured in caecum content, liver and spleen tissue, and both innate and adaptive immune responses were characterized from 4 to 28 days post infection. Significant differences in *S.* Enteritidis colonization in the various organs and in innate and adaptive immune responses were found between the different groups. Moreover, this study showed that birds infected at the age of one day are much more susceptible to an infection, than birds infected at the age of one week. However, animals at both ages showed a similar trend in response to the infection. Observations suggest that lower activity of the phagocytes and higher humoral activity prevents a systemic infection of *Salmonella* in liver and spleen. This study indicated that there is a relationship between various parts of the immune system in relation to the rate of (systemic) infection. Differences in bacterial load between groups

after an infection was further investigated, using five of the genetic groups tested for their natural resistance (chapter 4). These groups were infected with *S.* Enteritidis (**chapter 6**). The bacterial load in caecum content and liver and spleen tissue was measured shortly after infection from 4 to 10 days post infection, in order to quantify the differences in susceptibility between these groups. Considerable differences in *S.* Enteritidis load in the organs were found between the groups. Results showed that the group with the highest natural resistance (chapter 4) also showed the highest resistance to *Salmonella*, a similar finding to what we described in a previous experiment, chapter 5.

Data from the infection experiment described in chapter 6 were used to identify genes involved in genetic resistance to *S.* Enteritidis as described in **chapter 7.1**. For that purpose the candidate gene method is a suitable technique and was used here to investigate twelve candidate genes involved in the pathogenesis of *S.* Enteritidis infections, based on knowledge of studies in both man and chicken. Results illustrated that eleven of the twelve selected genes had a significant association with one of the phenotypic traits (*S.* Enteritidis load in caecum, spleen or liver). These results confirmed that *Salmonella* resistance is a polygenic trait, which makes it possible, but complex to breed animals that are resistant to *S.* Enteritidis. Another route to select new candidate genes is by finding differentially expressed genes as described in **chapter 7.2**.

Single genes or genes from pathways with the largest effects should be chosen when several genes are used in breeding activities. Breeding for disease resistance will result in a lower colonization level of animals and a higher threshold to become infected, through which the infection spreads much slower through a flock compared to the situation nowadays. It is important to make sure that there is no selection on undesired production traits together with the target trait, before using these in selection strategies. Further research needs to be conducted to identify more candidate genes, or pathways using for example micro arrays. With these data it should be possible to understand more about the mechanism of disease resistance, and what genes play a major role in disease resistance. To conclude, together with other strategies to control *Salmonella* that already exist, the genetic approach may provide additional means to control *Salmonella*.

# NEDERLANDSE SAMENVATTING

*Salmonella* is een bacterie die ziekte kan veroorzaken in mens en dier. Jaarlijks krijgen in Nederland ongeveer 50.000 mensen gastro-enteritis als gevolg van een *Salmonella* infectie. Gastro-enteritis bij de mens wordt gekenmerkt door diarree en braken. Een *Salmonella* infectie kan ernstige ziekte veroorzaken bij mensen, vooral ouderen en jonge kinderen kunnen aan een infectie overlijden. De meeste uitbraken van humane Salmonellosis worden veroorzaakt door pluimveeproducten (19% door kippenvlees en 34% door eieren), maar ook varkensvlees en rundvlees zijn belangrijke veroorzakers van *Salmonella* infecties (respectievelijk 25% en 14%), de overige 8% wordt veroorzaakt door andere bronnen. De meeste uitbraken zijn te voorkomen door het goed gekoeld bewaren, bereiden en verhitten van het voedsel, waardoor de bacterie geen kans krijgt zich te vermeerderen, en dood gemaakt wordt door het verhitten.

*Salmonella* veroorzaakt niet alleen bij de mens infecties, maar ook kippen kunnen besmet raken door verschillende *Salmonella* typen. Deze *Salmonella* infecties zorgen voor economische problemen in de pluimveehouderij. De mate van besmetting hangt af van verschillende factoren, zoals, de stam en het serotype van de bacterie, de leeftijd van de kippen, en de genetische achtergrond van de kippen. Jonge dieren (minder dan een week oud) zijn in het algemeen veel gevoeliger voor *Salmonella* infecties dan oudere dieren. Bij jonge dieren kan een infectie de dood tot gevolg hebben. Oudere dieren zijn veel beter bestand tegen infecties, ze kunnen wel geïnfecteerd zijn met *Salmonella* en de infectie overdragen, maar deze dieren vertonen vaak weinig of geen ziekteverschijnselen. Deze dieren zijn dan drager van *Salmonella*, en zijn een bron van besmetting voor andere dieren in de groep. De infecties in kippen worden vaak voorkomen met behulp van vaccins (inentingen) of behandeld met behulp van antibiotica. Echter, door het veelvuldige gebruik van antibiotica, bestaat het risico dat deze bacteriën resistent worden tegen deze antibiotica, waardoor het behandelen van de infecties in mens en dier veel moeilijker wordt. Het gebruik van vaccins is vaak kostbaar en vaccinatie beschermt niet altijd goed.

*Salmonella* infecties bij bedrijven kunnen voorkomen worden met een goede en hygiënische bedrijfsvoering zoals goede, afgesloten stallen, en voldoende hygiëne bij het binnenkomen en verlaten van de stallen. Maar het is moeilijk en kostbaar om al deze maatregelen zorgvuldig uit te voeren. Een andere mogelijkheid om de aantallen *Salmonella* infecties in kippen te verminderen is het fokken van kippen die meer resistent zijn tegen *Salmonella*. Met verhoogde genetisch resistente dieren zal de werking van het immuun systeem versterkt worden, waardoor het antibiotica gebruik kan worden terug gedrongen. Omdat deze dieren minder snel ziek worden, zal bovendien het dierenwelzijn verhoogd worden, wat tegenwoordig een belangrijk aspect is in de dierhouderij.

*Salmonella*, is een gram negatieve, facultatief intracellulaire bacterie. Een *Salmonella* infectie bij mens en dier start vaak na het eten van besmet voedsel, waarna de bacterie zich kan vestigen en vermeerderen in de darmen (koloniseren) en de

ziekteverschijnselen zich kunnen openbaren Vervolgens kan *Salmonella* door de darmwand heen dringen en zich daar vestigen, waarna het immuunsysteem geactiveerd wordt om de bacterie aan te pakken. Het immuunsysteem speelt dus een belangrijke rol bij genetische resistentie. Als *Salmonella* het lichaam is binnengedrongen komt als eerste de aspecifieke immuun respons op gang, in de vorm van fagocyterende cellen. Dit zijn cellen die allerlei lichaamsvreemde stoffen, waaronder *Salmonella*, aspecifiek kunnen opnemen, en vervolgens opruimen. Een andere taak van deze cellen is het activeren van het andere gedeelte van de immuun respons, de specifieke respons. De specifieke respons reageert, zoals zijn naam ook aangeeft, specifiek op een bepaald pathogeen, waarna er ook een geheugen gevormd vormt om een volgende infectie eerder en sneller de baas te kunnen (zie ook **hoofdstuk 1**). Om *Salmonella* infecties te voorkomen of te bestrijden via fokkerijmaatregelen om zo de kans op humane infecties terug te brengen is het dus belangrijk om dieren te selecteren die genetisch meer resistent zijn tegen *Salmonella*. De eerste afweer linie, de aspecifieke respons lijkt hierbij enorm belangrijk.

Het onderzoek in dit proefschrift beschrijft het ontwikkelen van genetische merkers voor functies van de fagocyterende cellen, en de rol van deze cellen in resistentie tegen *Salmonella* in vleeskuikens. Een genetische merker is een eigenschap (genotypisch of fenotypisch) waarmee de positie van een gen, of waar het gen in de buurt ligt kan worden bepaald. Met behulp van deze genetische merkers is het mogelijk om dieren op voorhand te selecteren op resistentie of gevoeligheid tegen *Salmonella*. Allereerst zijn hiervoor *in vitro* (= op het laboratorium) en *in vivo* (= in het dier) experimenten nodig om het aspecifieke en specifieke immuunsysteem te karakteriseren na een *Salmonella* infectie. In **hoofdstuk 2** is de belangrijke rol van bepaalde fagocyterende cellen van de aspecifieke respons, de macrofagen, beschreven na een *in vitro* infectie. Hierbij is de opname en overleving van *Salmonella* gemeten in macrofaag cellijnen en T- en B-lymfocyt cellijnen. Al deze cellijnen waren in staat om *Salmonella* op te nemen, en *Salmonella* was in staat om in al deze cellijnen te overleven, hoewel de effectiviteit van deze processen tussen de cellijnen enorm verschilden. De resultaten laten zien dat de macrofaag cellijnen in staat waren om de hoogste aantallen bacteriën op te nemen, waarna ze vergeleken met de lymfocyt cellijnen ook onmiddellijk en sneller begonnen met opruimen. Op basis hiervan blijken macrofagen het meest effectief te zijn in het opnemen en opruimen van *Salmonella*. Het belang van de aspecifieke response is ook *in vivo* onderzocht in een dierexperiment, waarbij dieren geïnfecteerd werden met *Salmonella* (**hoofdstuk 3**). Omdat de aspecifieke respons een belangrijke rol blijkt te spelen kort na een infectie, zijn er dieren gebruikt waarbij kort na infectie (tussen 0 en 3 dagen na infectie) verschillende immuun parameters zijn gemeten. Die resultaten lieten zien dat de functie van fagocyterende cellen verhoogd werd in deze periode na infectie. Dit kan komen omdat de er hogere aantallen cellen naar de plaats van

infectie gingen, of omdat de activiteit van deze cellen verhoogd was, wat niet is uit te sluiten.

Het immuunsysteem speelt een centrale rol in de afweer van *Salmonella* nadat een dier is geïnfecteerd. Het is bekend dat ieder individu, ieder dier, anders reageert op een infectie, soms wordt een dier ziek na een infectie maar soms komt een dier er goed doorheen zonder echt ziek te worden. Dit is afhankelijk van vele factoren, zoals stress, andere infecties, leeftijd en dergelijke, maar hierbij speelt ook de genetische aanleg een rol die het immuun systeem kunnen beïnvloedden. Om te kijken of verschillen die aanwezig waren in de basale immuun profielen (natuurlijke resistentie) zijn te relateren aan verschillen in respons na een infectie (ziekte resistentie) is een experiment uitgevoerd met 7 verschillende genetische groepen kippen (**hoofdstuk 4**). Er werden verschillende kenmerken gemeten van de aspecifieke en specifieke immuun respons van de dieren. De resultaten lieten zien dat er aanzienlijke verschillen aanwezig zijn in natuurlijke resistentie tussen de verschillende groepen. Geen van de groepen gaf de beste respons over alle gemeten immuun kenmerken, de ene groep was beter in de aspecifieke respons, de andere groep beter in de specifieke respons, of ergens er tussenin. Vervolgens is onderzocht of deze verschillen in de basale immuun respons gerelateerd konden worden aan ziekte resistentie tegen *Salmonella* bij vleeskuikens.

Daartoe is in een experiment de verschillen tussen vier groepen vleeskuiken in aspecifieke en specifieke immuun respons gemeten, en deze is vergeleken met de resultaten van mate van infectie in de organen na een *Salmonella* infectie (**hoofdstuk 5**). Het bleek dat de vier groepen in verschillende mate geïnfecteerd waren met *Salmonella*, en dat deze vier groepen ook verschilde in immuun respons. Dieren die geïnfecteerd waren op de leeftijd van één dag oud zijn veel gevoeliger voor *Salmonella* dan dieren geïnfecteerd op een leeftijd van een week oud, de mate van infectie (hoeveelheid bacteriën in een orgaan) van de dieren was hoger en het immuun systeem kwam later op gang. Maar er leek wel een vergelijkbare trend in immuun respons te zijn bij de dieren die op verschillende leeftijden geïnfecteerd waren. Een lagere activiteit van de aspecifieke immuun respons en een hogere activiteit van de specifieke immuun respons voorkomt dat *Salmonella* vanuit de darm naar de lever en milt kan gaan. Hieruit bleek dat er een verband bestaat tussen de verschillende delen van het immuun systeem en de mate van infectie in diverse organen.

Vervolgens is gekeken naar hoe de groepen dieren waarbij de basale immuun respons is gekarakteriseerd (hoofdstuk 4) reageren op een *Salmonella* infectie, om te onderzoeken of de verschillen tussen de groepen in basale immuun respons te relateren zijn aan de verschillen in ziekteresistentie (**hoofdstuk 6**). Hiervoor werden vijf groepen gebruikt die reeds eerder zijn onderzocht op natuurlijke resistentie (hoofdstuk 4), en deze groepen werden geïnfecteerd met *Salmonella* op 3 weken leeftijd. Kort na infectie werd de mate van infectie te bepaald in de organen, om de verschillen in gevoeligheid voor *Salmonella* te

bepalen tussen de groepen. Hieruit bleek inderdaad dat de groep met de hoogste natuurlijke resistentie (hoofdstuk 4) ook de meest resistente groep tegen *Salmonella* leek te zijn, wat overeenkwam met de resultaten beschreven in hoofdstuk 5.

De resultaten van het experiment uit hoofdstuk 6 werden tevens gebruikt om te onderzoeken welke genen er nu werkelijk een rol spelen bij genetische resistentie tegen *Salmonella* (**hoofdstuk 7.1**). Hierbij zijn twaalf verschillende genen onderzocht waarvan bekend is dat deze een belangrijke rol spelen bij een *Salmonella* infectie. Elf van de twaalf genen bleken een significante interactie te hebben met verschillen in gevoeligheid tegen *Salmonella*. Verder is in **hoofdstuk 7.2** nog een andere techniek beschreven, waarbij er via een andere route (namelijk, verschillen in de mate dat genen tot expressie komen) nieuwe kandidaat genen kunnen worden ontwikkeld.

De resultaten van onderzoek naar genetische resistentie tegen *Salmonella* en de karakterisering van genen die bij *Salmonella* resistentie een rol spelen zijn hoopvol voor de toekomst. Over enkele jaren zou er binnen fokprogramma's geselecteerd kunnen worden op dieren die een verhoogde resistentie hebben tegen infectieziekten. Als hierbij gebruik gemaakt wordt van genen met grote effecten, zal er ook grondig onderzocht moeten worden of deze selectie gepaard gaat met selectie op ongewenste kenmerken, voordat het werkelijk in de praktijk toegepast kan worden. Samen met andere strategieën om *Salmonella* infecties terug te dringen, kan de genetische resistentie in de toekomst een belangrijke bijdrage leveren in de beheersing en terugdringen van *Salmonella* infecties.

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**SUBMITTED:**

KRAMER, J., A.H. VISSCHER, J.A. WAGENAAR, J.B.J.W. CORNELISSEN AND S.H.M. JEURISSEN Comparison of natural resistance in seven genetic groups of meat-type chicken (submitted to British Poultry Science).

KRAMER, J., J.A. WAGENAAR, S.H.M. JEURISSEN, AND A.H. VISSCHER Genetic resistance to *Salmonella* enteritidis carrier-state of five different genetic groups of meat-type chicken (Submitted to Avian Pathology)

KRAMER, J., M. MALEK AND S.J. LAMONT Association of twelve candidate gene polymorphisms and response to challenge with *Salmonella* enteritidis in poultry (submitted to Animal Genetics)

# CURRICULUM VITAE

Op 12 mei 1975 werd ik, Judith Kramer, in het dorpje Ilpendam geboren. In 1993 heb ik mijn VWO diploma gehaald aan het GSG Helinium, te Hellevoetsluis. In dit zelfde jaar ben ik begonnen met de studie Bioprocestechnologie aan de Landbouwuniversiteit Wageningen (nu Wageningen Universiteit en Research Center). In september 1998 ben ik afgestudeerd met de specialisatie moleculair – cellulair. Vanaf oktober 1998 ben ik als Assistent in Opleiding (AIO) werkzaam geweest bij de Erasmus Universiteit Rotterdam, Faculteit der Geneeskunde en Gezondheidswetenschappen en was gedetacheerd bij de divisie Dier en Omgeving bij het instituut voor Dierhouderij en Diergezondheid (ID-Lelystad). Bij deze divisie, in samenwerking met andere groepen binnen het instituut, heb ik het onderzoek uitgevoerd dat heeft geleid tot dit proefschrift. Sinds 1 oktober 2002 ben ik als postdoc werkzaam bij het cluster Diergenomica voor de divisie Dier en Omgeving van ID-Lelystad.