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Genetic susceptibility for Salmonella infections

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ABSTRACT

Genetic susceptibility to Salmonella infections

The Salmonella species Typhimurium and Enteritidis form the most important causes of food poisoning. Immunity to Salmonellae requires innate and specific immune responses. Reported here are the genetic polymorphisms in the genes of Nramp1, Toll-like receptors and CD14 related to the innate immune response to Salmonellae. Salmonella species are typically associated with the intra-cellular pathogens capable of surviving and replicating intra-cellularly in the phagocyte. Consequently, an adequate T-lymphocyte type 1 response is required to eliminate the parasite. The genetic factors that determine the susceptibility of the host to Salmonella infection are described below. Mutations in the human genes of some crucial cytokines of the type 1 pathway, like IFN-, IL-12 and IL-18, greatly reduce the natural resistance to Salmonella infections. Mutations in the human genes of this type 1 pathway are, by definition, seldom found in humans. By investigating the more frequently occurring (more than 1% of the population) polymorphisms in type 1 cytokines, and those of the innate immune response, one can assess the relative risk of genetic susceptibility at population level. In conclusion, it is feasible and useful to perform population studies on the effect of genetic polymorphisms on the susceptibility of the host. Such studies, not described to date, are important in the risk assessment of Salmonellae food poisoning. Suggestions and recommendations are presented here for studying the genetic factors in the host resistance to salmonella infections in human and animal models.

Key words: Salmonella, food poisoning, resistance genes, genetic polymorphism, susceptibility

RAPPORT IN HET KORT

Genetische gevoeligheid voor Salmonella infecties

Contaminatie van eieren en vlees met Salmonella met Campylobacter bacteriën is de belangrijkste oorzaak van voedselvergiftiging en de kans op zo'n voedselvergiftiging wordt mede bepaald door de genetische achtergrond van de gastheer. Dit rapport geeft een overzicht van humane- en dierstudies naar deze genetische gevoeligheid van de gastheer voor Salmonella-bacteriën.

De immunologische afweer tegen Salmonella bestaat uit een niet-specifiek en een specifieke deel. Voor het afdoende couperen van een Salmonella-infectie is een adequate T-helper type 1 (Th1) respons (behorend tot de specifieke immuunrespons) noodzakelijk en cruciale eiwitten in deze Th1-route zijn IFN-γ, IL-12, en IL-18. Net als mutaties in genen die bij de niet-specifieke immuunrespons betrokken zijn, zoals Nramp1, 'Toll-like' receptoren en CD14, verhogen mutaties in de genen van deze Th1-eiwitten de gevoeligheid voor Salmonella-infecties.

Mutaties zijn echter zeldzaam. DNA variaties (polymorfismen) komen daarentegen vaker voor, namelijk bij meer dan 1 procent van de bevolking. Dergelijke variaties leiden tot een kleine verandering in de structuur of expressie van het eiwit, waardoor de effectiviteit van de afweer tegen Salmonella-bacteriën wordt veranderd. De effecten van deze polymorfismen op de immuunrespons na een voedselvergiftiging zijn weliswaar subtiel, maar op populatieniveau kan hun 'impact' aanzienlijk zijn.

Trefwoorden: Salmonella, voedselvergiftiging, resistentie-genen, genetische polymorfismen, infectie-gevoeligheid

Contents

List of abbreviations

CD (Cd)	cluster of differentiation
IFNγ	Interferon gamma
IL	interleukin
Lbp	lipopolysaccharide (LPS) binding protein
NADPH	nicotinamide dinucleotide phosphate
Nramp	natural resistance-associated macrophage protein
NOS2	nitric oxide synthase type 2, iNOS
ROI	reactive oxygen intermediates
RNI	reactive nitrogen intermediates
SCV	salmonella containing vacuole
Th1	T helper 1
TNFα	tumor necrosis factor-α
TLR	Toll-like receptor
Xid	X-linked immunodeficiency
XLA	X-linked agammaglobulinemia

SAMENVATTING

De Salmonella species Typhimurium en Enteritidis zijn de belangrijkste oorzaken van voedselvergiftiging. De genetische factoren, die de susceptibiliteit van de gastheer voor salmonella-infecties bepalen, worden in dit rapport beschreven.

De immunologische afweer tegen Salmonellae bestaat uit de innate en de specifieke immuun respons. Met betrekking tot de innate respons tegen Salmonellae, zijn genetische polymorfismes in de genen van Nramp1, 'Toll-like' receptoren en CD14 gerapporteerd.

Een typische eigenschap van Salmonellae is, dat zij behoren tot de (intra-cellulaire) pathogenen, die intracellulair in de fagocyt kunnen overleven en delen. Dientengevolge is een adequate T-lymfocyte type 1 respons noodzakelijk om de parasiet te doden. Mutaties in de humane genen van enkele cruciale cytokines in deze route, zoals IFN-γ, IL-12, en IL-18, verlagen sterk de natuurlijke weerstand tegen salmonella-infecties. Mutaties in de humane genen van deze type 1 cytokines komen, per definitie, zelden voor. Door de vaker (bij meer dan 1% van de populatie) voorkomende polymorfismes in de type 1 cytokine genen, alsmede die van de innate immuun respons te onderzoeken, is het relatieve risico van genetische gevoeligheid voor Salmonellae op populatieniveau te bepalen.

De conclusie is, dat het mogelijk en aantrekkelijk is om populatiestudies uit te voeren teneinde het effect van genetische polymorfismes op de gevoeligheid van de gastheer voor salmonella-infecties te bepalen. Dergelijke studies werden tot op heden niet of nauwelijks uitgevoerd en de kennis die dit oplevert is onder andere van belang voor de risico analyse van voedselvergiftiging met Salmonellae**.**

SUMMARY

The Salmonella species Typhimurium and Enteritidis are the most important causes of food poisoning. The genetic factors that determine the susceptibility of the host to salmonella infections have been presently described.

The immune defence against Salmonellae requires innate and the specific immune responses. With respect to the innate immune response to Salmonellae, genetic polymorphisms in the genes of Nramp1, Toll-like receptors and CD14 were reported.

Salmonella typically belong to the intra-cellular pathogens that are capable to survive and replicate intracellularly in the phagocyte. Consequently, an adequate T-lymphocyte type 1 response is required to eliminate the parasite. Mutations in the human genes of some crucial cytokines of this pathway, like IFN-γ, IL-12, and IL-18, greatly reduce the natural resistance to Salmonella infections. Mutations in the human genes of this type 1 pathway are, by definition, seldom found in human. By investigating the more frequently (in more than 1% of the population) occurring polymorphisms in type 1 cytokines and those of the innate immune response, one may assess the relative risk of genetic susceptibility at population level.

It is concluded that it is feasible and useful to perform population studies to the effect of genetic polymorphisms on the susceptibility of the host. Such studies have not been described to date and are important in the risk assessment of Salmonellae food poisoning. Suggestions and recommendations are therefore given to study the genetic factors in the host resistance to salmonella infections in humans and animal models.

1. INTRODUCTION

In humans, *S*. Enteritidis (*Salmonella enterica enterica* serovar Enteritidis, SE) and *S.* Typhimurium cause food poisoning. Other important food-borne pathogens are *Campylobacter jejuni, E. coli* O157:H7 and *Listeria*. In contrast, *S.* Typhi and *S.* Paratyphi are transmitted through human waste and cause typhoid fever. Despite its declining incidence, *S.* Typhi infection remains an important health threat, mainly for people living in developing countries with more than 16 million cases of disease and 600,000 deaths annually. As the topic of this review is to evaluate the importance of genetic factors in salmonella food poisoning, the presentation of data will focus on the species *S*. Enteritidis and *S.* Typhimurium.

The Gram-negative bacterium *Salmonella enterica subspecies enterica* belongs to the family *Enterobacteriaceae*. The subspecies Salmonella *enterica enterica* comprises almost 4000 serotypes, all more or less pathogenic for humans. These Salmonella types are genetically quite similar with serotype differences based on surface antigens such as LPS and flagella.

S. Typhimurium has a broad host-range and is transmitted from animals to humans via the consumption (at least in the Netherlands) of (faecally) contaminated meat products from cattle, pigs and poultry and notably eggs [2]. Consumption of contaminated eggs is the major reason for *S.* Enteritidis infections. Due to the centralised and wide-scale distribution of manufactured foods (cases of contaminated ice cream, milk powder, pasteurised milk), salmonella infections are rapidly increasing and have meanwhile emerged to a worldwide pandemic of food poisoning. In the Netherlands and most other industrialised countries, as a result of veterinary measures, improvements in slaughter hygiene and food processing, the incidence of salmonella food poisoning is now declining (cf. Fig. 1.). Still, some 50,000 cases of salmonella food poisoning are reported in the Netherlands annually [3] of which around 50-60 cases are fatal [2]. These infections occur both sporadically (some 60-80% of the cases) and also as part of larger outbreaks. It is thought that in industrialised countries less that 1% of these infections is clinically notified. In 2003, there were 2142 confirmed cases of salmonella in the Netherlands (incidence 20.7/100,000). The number of patients looking for medical care and visiting their general practitioner is approximately 2.5x higher. While it is estimated that in the general population the incidence of salmonella is at least 14.5 times higher than laboratory confirmed cases.

The severity of these infections (and consequently the tendency for reporting) is dependent on the serotype, the infective dose and also the host response. Though the overwhelming majority of salmonella infections show a subclinical course, clinical symptoms may arise that vary from

an acute self-limited gastroenteritis to typhoid fever and even life-threatening septicemia (blood poisoning). Gastroenteritis can be quite debilitating in the very young, the very old, and the immune-compromised, and causes significant morbidity and mortality.

In comparing the results obtained in animal and humans exposed to Salmonellae, it is important to note that *S.* Typhimurium and *S.* Enteritidis cause gastroenteritis in humans, yet causes a typhoid-like disease in rodents.

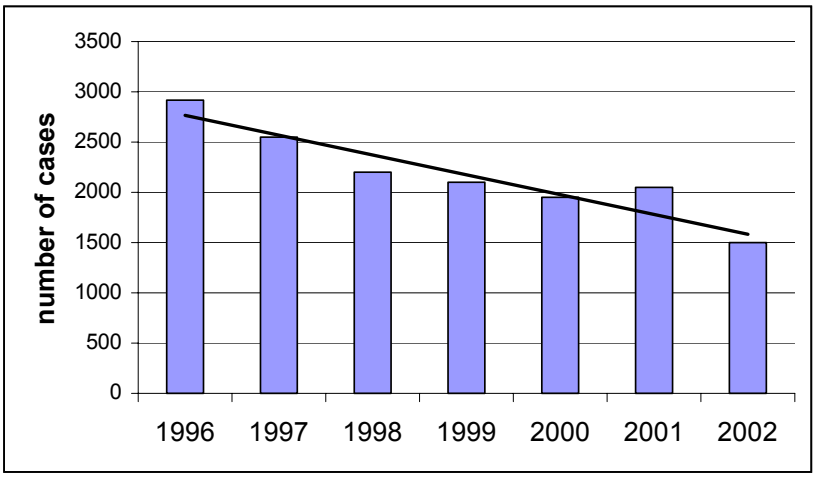

Figure 1. Number of reported cases of human salmonella infections in the Netherlands. Source: Dutch Meat Board, based on RIVM-data.

As will be outlined in this report, the severity and outcome of salmonella infections depend on the combination of the "virulence" of the infecting strain, the dose, the immune status of the host, and the genetic make-up of both bacterium and the host. This report reviews the resistance against salmonella infection in relation to the genetic background of the invaded host. In addition, the host-pathogen interactions and the validity of mice models for salmonella infections in humans will be addressed.

2. PATHOGENESIS OF SALMONELLOSIS

In cases of food poisoning, Salmonellae is rapidly transferred to the acid stomach, which forms the first step of defence against Salmonella. This acid-barrier of the stomach inactivates Salmonellae, because these bacteria do not resist to low pH (<3). Once in the intestinal tract, salmonella has to adhere to intestinal epithelium, but this attachment is counteracted by peristalsis, secretory IgA-antibodies, defensin peptides, mucins from Goblet cells, and commensal bacteria (microflora) competing for the same binding sites.

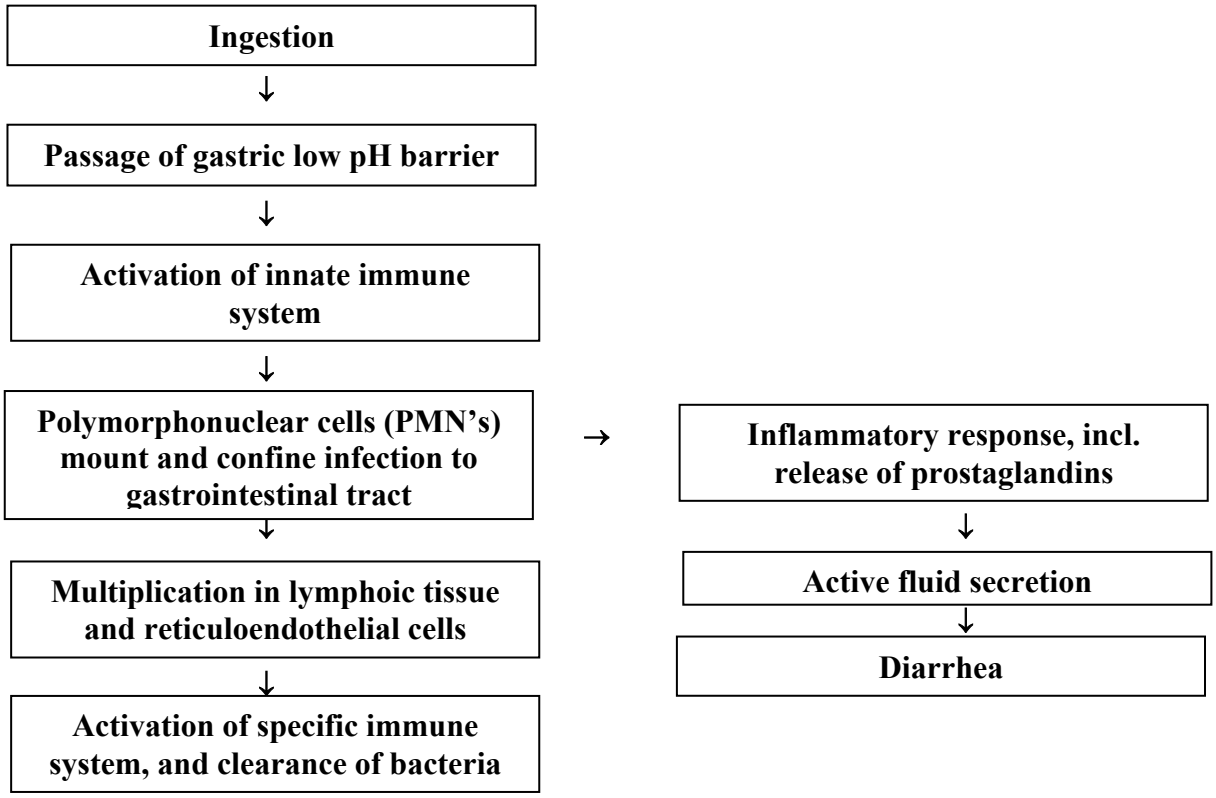

Figure 2. Schematic pathogenesis of Salmonellosis in man.

Depending on the serovar or the species of the infected host, Salmonellae remain mostly localised to the intestinal epithelium and the gut associated lymphoid tissues (Peyers plaques). In rare cases, Salmonella becomes systemic and invades deeper tissues. In subclinical infections, the innate immune system controls progression to disease. In humans, once beyond the epithelial barrier, four following phases of a clinical infection can be recognised:

1. Activation of the innate immune system with at first the influx of polymorphonuclear cells (neutrophils), followed by mononuclear cells (monocytes and macrophages) that partly internalise the bacteria via phagocytosis and release various cytokines, like IL-8, TNFα and

IFN-γ (cf. Fig. 3). As the inflammatory reaction progresses, neutrophils migrate from the lamina propria through the epithelial layer, with accumulation of inflammatory cells and protein-rich fluid into the intestinal lumen. This causes epithelial detachment from the basal membrane, favouring fluid secretion into the intestinal lumen and diarrhoea;

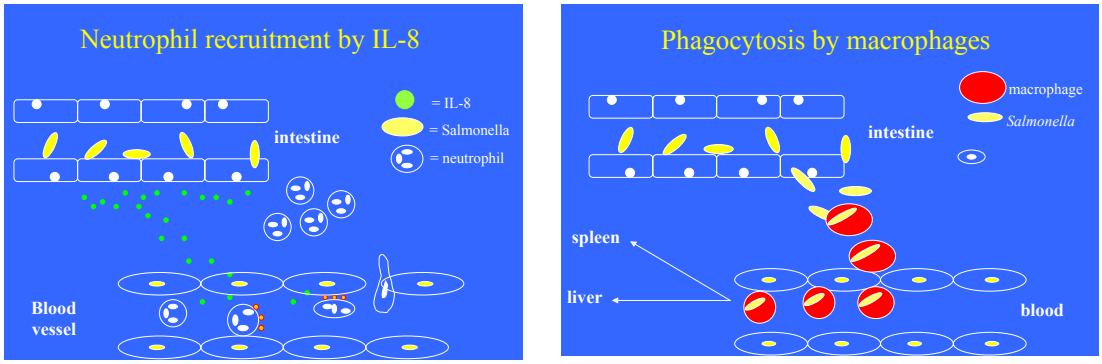

Figure 3. Influx of neutrophils and clearance of Salmonella bacteria by macrophages.

2. Following phagocytosis, intracellular and exponential growth of the bacteria, since Salmonella are able to counteract the anti-microbial defence of macrophages. In the infected macrophages, the Salmonella survive and replicate. As such, infected macrophages serve as a Trojan horse, facilitating the spread of Salmonella to the mesenteric lymph nodes, liver and spleen and circulation. Those bacteria that appear in the circulation are rapidly internalised (phagocytosis) by monocytes and neutrophils;
3. Activation of the specific immune response; T and B cells encountering Salmonella antigens in lymph nodes become activated. This response is crucial to eliminate Salmonella;
4. The final and fourth phase is clearance of the bacteria. Activated T-cells produce type 1 cytokines that activate the infected macrophages. Once activated, the macrophages are able to kill the intracellular bacteria (i.e. Salmonella) and the infection is cleared.

In summary, in salmonella-gastroenteritis, neutrophils and phagocytes in the lamina propria usually inactivate Salmonellae, the infection is usually self-limiting with some diarrhoeal symptoms and does not proceed beyond the lamina propria. However, under certain conditions (immune-compromised host, high pH in stomach of elderly, high bacterium load or a highly virulent strain) the immune system looses control of the infection. The phagocytosed bacteria survive the killing by oxygen radicals and show intracellular growth within the phagocytes. The infected phagocytes then serve as a way of transport, gain access to the lymphatics and bloodstream permitting a secondary bacteraemia with spread to the liver, spleen and the biliary

tract [4,5]. Bacteria multiply in the biliary tract which leads to seeding the intestine with large numbers of bacteria and gives a fatal sepsis in 1 to 4% of the cases (mostly due to some form of immunodeficiency).

As compared to humans, the course of infection in rodents is different. In the mouse model the course of a sublethal infection evolves the following five stages: (1) Salmonellae pass the lamina propria; (2) clearance from the blood; (3) early exponential bacterial growth of the remaining bacteria in reticuloendothelial system (RES), mainly monocytes; (4) adaptive response with suppression of growth by activated macrophages and NK-cells with IFN-γ and TNFα as mediators, and (5) clearance from the tissues, which requires T-cell dependent immunity [6].

3. THE IMMUNE DEFENSE AGAINST SALMONELLAE

Microorganisms are cleared from the host via the non-specific (innate) immune defence and the specific immune defence. The innate immune system primarily represents the front-line defence (first step) and comprises mucosal integrity; complement, and phagocytes. Phagocytes are either polymorphonuclear cells (PMNs; neutrophils) or mononuclear cells (monocytes and macrophages), and kill the engulfed pathogen by lysosomal enzymes and toxic reactive oxygen (ROI) and reactive nitrogen intermediates (RNI). The bacterium is degraded into fragments that are presented as antigens to T-cells (antigen presentation).

In a later stage, the specific immunity (cell-mediated and antibody-mediated immunity) is induced in a later stage by antigen-presenting cells (dendrites, macrophages). In response to presentation of the microbial antigens, Th-lymphocytes proliferate and secrete cytokines, which activate macrophages. In addition, B cells are triggered by bacteria and activated T-cells to produce immunoglobulins, like IgA, that, in addition to complement factors, opsonise the surviving bacteria (bind to bacteria thereby facilitating phagocytosis).

A special feature of Salmonella (as well as *Mycobacterium tuberculosis* and *Listeria monocytogenes*) is that it belongs to the class of facultative intracellular pathogens. Intracellular pathogens are resistant to intra-phagocytic (intracellular) killing, so that they can survive and even replicate within monocytes and macrophages (intracellular survival and growth). Evidently, these bacteria are resistant to the innate immune defence system and the activation of cellular immunity is urgently required to prevent a systemic and lethal infection (septicaemia, sepsis). Indeed, T-cells with a central role in cell-mediated immunity, induce an adequate type 1 response to combat the pathogen at this stage (intracellular survival and growth of Salmonellae in the macrophages).

Certain cytokines, released from neutrophils and macrophages, drive the T-lymphocytes to the T helper-1 (T_h1) phenotype that produce and release the so-called type-1 cytokines, like IL-12, IL-18, but in particular IFN-γ, which further activates the bacteria-loaded macrophages. These activated macrophages will then clear the intracellular pathogens not eliminated by non-activated macrophages.

4. GENETIC SUSCEPTIBILITY IN HUMANS

Genetic control of immunity to infections is complex, because the host-pathogen recognition and killing of the pathogen that determine the outcome of infection consists of multiple steps, implicating that host susceptibility to infection is under multigenic control. Genetic alterations in host resistance genes vary from null mutations leading to cause complete loss of function, to silent mutations with no change in function or to mutations with a novel or altered function. If the incidence of the mutation in the population is higher than 1%, the mutation is described as a polymorphism.

Mutations that induce the missing of a crucial link in an essential pathway of the immune defence are highly penetrant, but have a low incidence. They manifest early in life and are often lethal. An example of such a rare but functional mutation is found in humans that suffer from severe infections due to (otherwise weakly pathogenic) intracellular pathogens, like Salmonella, non-tuberculous mycobacteria or Mycobacterium bovis bacille Calmette-Guerin. Mutations in five genes encoding essential proteins of the type 1 cytokine cascade have been found in these patients: complete and partial IFN-γR1 deficiency, a null mutation in IFN-γR2 (encoding the IFN-γ receptor signalling chain), a large deletion in IL-12p40 (encoding the p40 subunit), mutations of IL-12Rbeta1 chain (encoding the beta1 subunit of the IL-12 receptor), and STAT1. This topic is reviewed by Ottenhoff et al. [7], and they showed that the incidence of defects (i.e. mutations) in the type 1 pathway is very low. The more frequently occurring polymorphisms in the genes of the type 1 cytokines (incidence > 1% of the population) may be more relevant for the susceptibility for salmonella infections at population level.

It is of interest, that salmonella infections were far more common among individuals with IL-12Rb1 receptor chain or IL-12 deficiency (about 90%), while this infection occurred in only a minority of patients with IFN-γ receptor deficiency (about 10%) [8-10]. It would, however, not be surprising if defects in other components of the type 1 cytokine axis will be identified in the future [10].

Results from animal studies (cf. the following sections), indicate that genetic defects in the host-pathogen interaction may lead to increased susceptibility to salmonella infections. These defects may vary from alterations in the entry mechanism (attachment, entry, recognition) to impaired activation of the innate and specific immune response. Relevant candidates, in addition the five previously mentioned genes, include: NRAMP1, TNFα, IL-12p35, IL-15, IL-23, IL-18 receptor, and the Toll-like receptor homologues [10-12].

5. SALMONELLA VIRULENCE FACTORS

Despite the pH-barrier in the stomach (cf. paragraph 2), pathogenic *Salmonellae* may survive this passage, considering that the pH in the stomach usually increases following food consumption, allowing these bacteria to reach the distal ileum and ceacum. All invasive infections start in one way or the other by passing the epithelial lining of the intestinal mucosal surface. Salmonella invades the Peyer's patches via M-cells (certain intestinal epithelial cells) that function as antigen sampling cells, but damaged tips of villi of enterocytes may also be an entry for Salmonella [13].

After passage of the epithelium, granulocytes, like neutrophils and macrophages, will eliminate the pathogen via stimulation of NADPH-oxidase and nitric oxide synthase (iNOS), which generate the potent antimicrobial oxygen and nitrogen radicals (ROI's and RNI's, respectively) [14,15]. The activity of iNOS is upregulated by TNF-α, IFNγ, IL-12, and IL-18 [16], but the precise mechanism of bacterial killing in the phagocytes remains unclear.

Salmonella bacteria have, however, developed multiple strategies to circumvent the bactericidal activities of ROI and RNI [17]. For instance, *S.* Typhimurium is able to exclude the NADPH-oxidase and iNOS in the vesicle where it resides. In addition, Salmonella is able to block activation of macrophages by inducing an increase in the production of the anti-inflammatory cytokine IL-10 [18], enabling the bacterium to proliferate in macrophages. Both the formation of vacuoles and IL-10 depend on virulence genes located on a second pathogenicity island, SPI-2 [18,19]. *S.* Typhimurium is also able to delay acidification of the SCV, promoting its survival.

With respect to the pathogen itself, special regions of the Salmonella genome, the so-called Salmonella Pathogenicity Islands (SPI's) encode the virulence factors of the initial stages of salmonellosis, including the onset of diarrhoeal symptoms. SPI-1 therefore controls the uptake and invasion of epithelial cells, induction of neutrophil recruitment, secretion of intestinal fluids, and partly the activation of specific and non-specific immune responses [19-21]. Entry into macrophages [22] and neutrophils [19] may also occur via SPI-1 mediated invasion, or via phagocytosis. Salmonella is also able to induce programmed cell death of infected macrophages, which presumably is an important mechanism for cell-to-cell spread. This is realised in at least two ways: apoptotic (delayed) and necrotic (rapid) cell death that respectively involve Salmonella SPI-1 encoded effectors, and SPI-2 and an outer membrane protein, regulated by IL-1β and IL-18 [23]. The function of SPI-2 is required for later stages of

infection, i.e. systemic spread and colonisation of host organs, including the replication in macrophages.

Proteins located outside SPI-2 (but secreted by the SPI-2 encoded secretion system) possibly contribute to the different host ranges of *S. enterica* serovars [24,25], since genomic loci encoding for these proteins show a variable distribution among the serovars and determine the pathogenicity of *S. enterica* serovars. Host specificity of the entrance of the pathogen is further mediated by outer membranous structures (fimbriae), whereas specific complement receptors (CR's) on macrophages of the host are involved in the recognition of Salmonella serovars. Interestingly, human macrophages recognise *S.* Typhi and *S.* Typhimurium by respectively CR-1 and CR-3, whereas murine macrophages recognise these strains by respectively CR-3 and CR-1. This finding suggests that the intracellular fate of Salmonella depends on the type of receptor involved in their recognition, and that CR-1 mediated recognition is related to intracellular survival [26].

6. SUSCEPTIBILITY IN MICE

In humans, infection with *S.* Typhimurium and *S.* Enteritidis usually induces mild symptoms, whereas oral challenge with Salmonella induces a severe infection in mice. Still, mice are suitable to study the mechanisms of salmonella infection, especially the first steps of an infection: adhesion and the subsequent entry. The growth of Salmonella results in high numbers of bacteria in liver and spleen, and the bacterial load can be used to quantify virulence and immunity. Secondly, various mouse strains are available that allow to investigate the relation between genetic background and susceptibility and infections. Table 1 shows an overview of inbred and wild type mice strains that differ in susceptibility to a salmonella infection.

Table 1. Salmonella susceptibility of various mouse strains.

Mouse strains	Mouse type
Inbred strains	
Extremely resistant	129S6/SvEvTac
Intermediate resistant	A/J
Extremely susceptible	C57BL/6J, BALB/c, C3H/HeJ
Wild type mice	
Resistant	CAST/Ei
More susceptable	MOLF/Ei, SPRET/Ei

7. SALMONELLA RESISTANCE GENES

Results from studies in rodents (knock-out mice, inbred strains differing in susceptibility to Salmonella, due to a mutation; cf. the previous paragraph) have shown the importance of various salmonella resistance genes and some will be reviewed below.

7.1 Nramp1

Nramp1 (natural resistance-associated macrophage protein 1; Slc11a1 [27,28]), found most abundantly in circulating monocytes/macrophages and PMN's, plays a key role in the resistance to intracellular pathogens in mice and man (reviewed by [29]). Nramp1 indirectly regulates delivery of lysosomal enzymes and codes for divalent cation transporters such as a pH-dependent manganese transporter [30]. Removal of these divalent cations by Nramp1 impairs the intraphagosomal (i.e. intracellular) microbial replication in the reticoloendothelial system (RES) [30]. In addition, Nramp1 regulates macrophage activation via the production of nitric oxide, IL-1β, INFγ, and MHC class II expression and Th1/Th2 differentiation [31].

Table 2. Genes involved in the resistance to salmonella infection.

Gene/factor	Name	Susceptibility*
Nramp1	Natural resistance-associated	↑↑↑↑
TLR4 (LPS)	Toll-like receptor for LPS	↑↑↑↑
btk (xid)	Bruton's tyrosine kinase	↑↑↑↑
Lbp	LPS binding protein	↑↑↑↑
CD14	High affinity receptor for LPS	↑↑↑↑
Enzymes for bacterial killing		
NADPH oxidase	$gp91^{phox}$, $p47^{phox}$, $p67^{phox}$, $p22^{phox}$	↑↑↑↑
NOS2	Nitric oxide synthase	↑↑↑↑
Cytokines		↑↑↑↑
Tnf	TNFα, tumor necrosis factor	↑↑↑↑
Tnfrsf1a	receptor TNF-Rp55	↑↑↑↑
Tnfrsf1b	receptor TNFpr75	↑↑↑↑
INFγ		↑↑↑↑
IL-12		↑↑↑↑
IL-12a	IL-12p35	↑↑↑↑
IL-12b	IL-12p40	↑↑↑↑
MIF	Macrophage inhibiting factor	↑↑↑↑

* Effect of deficiency/mutation

Mutations in the Nramp1 gene have been shown to be associated with an impaired resistance to a number of facultative intracellular pathogens, including Salmonella serovar Typhi in inbred mice [28,32]. A single mutation [33] gives complete loss of function of Nramp1 resulting in earlier death of the infected mice [34]. Mice showed a lower IFNγ gene expression and a delayed IFNγ response [35]. One study in six inbred chicken lines showed the presence of the G696A amino acid substitution in the coding portion of Nramp1 only in the chicken line, that was susceptible to *Salmonella enterica* serovar Typhimurium [36]. Two other studies showed, however, that genetic resistance of chicken to salmonella is not linked to Nramp1 mutations [37,38].

Several polymorphisms have been identified within the human homologue of Nramp1 (NRAMP1, localised to chromosome 2, 2q35), and some NRAMP1 polymorphism have been shown to be associated with intracellular pathogen infections (mycobacteria and tuberculosis), but not with Salmonella typhi induced typhoid fever in humans in Vietnamese man (exposure to *S.* Typhimurium was not evaluated) [39]. For instance, a 4-base pair deletion in the 3' untranslated region (UTR) [40] was significantly associated with tuberculosis in humans.

7.2 Toll-like receptors

TLR4 (Toll-like receptor 4; receptor for LPS, the typical component of Gram-negative bacteria) and TLR5 (receptor for flagellin; reduced expression/polymorphism in susceptible mice) have been proposed as key pathogen recognition factors that affect susceptibility to salmonella. Flagellins of several bacterial species are potent activators of the human innate immune system by binding to TLR5. Activation of TLR4 by LPS leads to the activation of the innate immune response and involves various host defence genes including pro-inflammatory cytokines such as TNFα [41], IL-1, IL-6, IL-8, and IL-12, chemokines, co-stimulatory molecules (CD80 and CD86), MHC class II and NOS2 by antigen presenting cells. Induction of CD80/CD86 and IL-12 by TLRs contributes to the initiation of adaptive immunity and the induction of Th1 effector responses.

TLR4 mutations found are a missense mutation in C3H/HeJ mice [42] and deletions in C57BL/10ScCr and C57BL/6.KB2 mice, all resulting in hyporesponsiveness to LPS and increased susceptibility to Salmonella. Overexpression of TLR4 was shown to be linked to a higher resistance to infection with Salmonella enterica serovar Typhimurium in chickens (likelihood ratio test of 10.2) [43]. Similarly in mice, over-expression of TLR4 amplified the host response to LPS and increased the survival of the mice in the early phase of salmonella

infection, but elicited a fatal and excessive inflammatory response in the later phase (>14 days) [44]. Survival was higher in Nramp1^{gly169} and TLR4 transgenic animals [44] and the combined effect of TLR4 and Nramp1 was synergistic (not additive). In humans, a TLR4-mutation (Asp299Gly) with a frequency of 3% to 11% was described that was associated with hyporesponsiveness to LPS [45] and a higher prevalence of gram-negative septic shock. The relevancy of TLR4-polymorphisms for salmonella infections in man is yet unclear.

7.3 LPS-binding protein and CD14

The innate defence against Salmonella Typhimurium involves binding of LPS to the CD14-receptor on monocytes and granulocytes. Lbp (LPS-binding protein), an acute phase protein, accelerates binding of LPS to CD14 and is essential for a rapid inflammatory response. CD14 (as well as TLR4 and lbp) deficient mice are therefore extremely resistant to the effect of LPS, show a large decrease in the expression of TNFα and IL-6 [46], and are highly susceptible to salmonella infection [47,48].

7.4 Bruton's tyrosine kinase

B-cells are important for the resistance to salmonella infections. For instance, a role of B cells in the susceptibility for Salmonella was demonstrated in mice with a defective B cell function (xid-mice, X-linked immunodeficiency) [49].

In humans mutations in the Bruton tyrosine kinase (BTK) gene causes the X-chromosome linked agammaglobulinemia (XLA), and this immunodeficiency is characterised by a deficiency of B lymphocytes, near absence of serum immunoglobulin, and recurrent bacterial infections. Indeed, salmonella infections have been described in XLA patients as well [50], though the most prominent symptoms of B cell immune deficiencies are respiratory manifestations.

7.5 NADPH and NOS2

Following phagocytosis of virulent Salmonella, the pathogen in the phagosome is killed by ROI and RNI (cf. section Entry). Animals deficient in either NADPH-oxidase or iNOS deficient show an increased susceptibility to salmonella infection [15,17]. Similarly, NOS2 knock-out mice can control early replication of Salmonella in the RES, but not later bacterial growth. Patients deficient in phagocyte NADPH-oxidase are susceptible to recurrent microbial

infections, including salmonellosis [12]

7.6 Cytokines

Like the human data (cf. section Genetic susceptibility to intracellular bacteria in humans), animal studies show that the successful host defence against Salmonella requires the type 1 response that involves IFN-γ and IL-12 (reviewed by [51-53].

The IL-12 p70 heterodimer is composed of two subunits: IL-12p35 (encoded by il12a) and IL-12p40 (encoded by il12b) [54]. In contrast to the p35 subunit, which is ubiquitously (constitutively) expressed in various cells including macrophages, expression of p40 subunit is highly regulated and is expressed primarily by macrophages and dendritic cells [54]. IL-12 binds to high-affinity beta1/beta2 heterodimeric IL-12 receptor (IL-12R) complexes on T cell and natural killer cells. IL-12 is produced and secreted mainly by dendritic cells, neutrophils and (infected) macrophages, and it has become evident that IL-12 skews T-cells to the Th1 phenotype eliciting IFNγ release from these T-cells, NK-cells and macrophages during intracellular infection [10].

The release of IL-12 from macrophages, particularly bioactive IL-12p70, and IFN-γ is under tight control; salmonella infection leads to increased IL-12p40 expression in Peyer's plexus, mesenteric lymph nodes, spleen, liver, while p35 is not affected [51]. In summary, IL-12 is a critical link between the innate and adaptive cell mediated immunity, capable of Th1 differentiation and IFNγ release by macrophages, T and NK cells.

The IL-12 receptor (IL-12R) is expressed by both NK-cells and by activated T cells. IL-12R is made up of two chains called IL-12Rb1 and IL-12Rb2, respectively [55]. Both receptor chains have extracellular, transmembrane and intracellular segments, which can bind IL-12 with low affinity. When co-expressed, IL-12 is bound with high affinity, initiating high IFN-γ production by T cells and NK cells. Note that STAT-4 is involved in (required for) a proper signal transduction following IL-12 receptor activation by the cytokine.

IFN-γ is another important cytokine as it has pleiotropic actions on a number of cell types, with the ability to modulate the function of over 200 genes. IFNγ is a homodimeric cytokine that binds to a heterodimeric receptor composed of two chains: IFNg-R1 (the ligand binding chain) and IFNg-R2 (the signalling chain, required for signal transduction) that are ubiquitously expressed [56]. IFN-γ is secreted mostly by macrophages, activated T cells and NK cells following IL-12 stimulation, and plays a key role in Th1 responses. Key actions of IFN-γ further include activation of macrophages, increased production of MHC class I and class II

proteins, activation of the cellular and humoral response via IgG heavy chain switching, upregulation of iNOS, modulation of the production of cytokines like IL-12, IFNγ itself and TNFα. IFN-γ interacts with a specific cell surface receptor, which is widely expressed on most nucleated cells.

TNFα is primarily produced by macrophages, activated NK cells and Th1 lymphocytes. TNFα acts synergistically with IFN-γ to activate neutrophils, macrophages and NK cells. TNFα exerts its effects via two types of receptors TNF-Rp55 (Tnfrsf1a, TNF receptor superfamily 1a gene) and TNFRp75 (Tnfrs1b). Mice without Tnfrsf1a showed an early susceptibility for Salmonella, due to their inability to target NADPH phagocyte oxidase harbouring vesicles to SCVs [57]. Rodent studies using live infection models have shown that neutralisation or gene deletion for TNFα is frequently associated with reduction of host defence in models of live Gram-negative infections, including salmonella infections [58].

Il12b knock-out mice (and less so Il12a knock-out mice) are susceptible to salmonella infection, because of the induction of a Th2 response, that is unable to eradicate the infection. Even attenuated Salmonella strains induce severe systemic infections in mice deficient in T-cells, IL-12, IL-18 or IFNγ-receptors [16,54]. Mice deficient in IL-12 lacked TNFα and IFN-γ responses [54], and mice deficient in either IL-18, a cytokine with IFNγ-inducing properties, or in STAT1 also display impaired Th1 responses to mycobacterial infection [59]. The role of ROIs should in this respect not be neglected, because ROI-scavengers completely abolished the IFNγ stimulatory effect [60]. B-cells are also important for the protective Th1 response, as T-cells produced less IFNγ in B-cell deficient mice [61].

Finally, macrophage migration inhibitory factor (MIF), produced by T cells, macrophages and intestinal epithelial cells, belongs to the Salmonella susceptibilty genes. MIF inhibits macrophage migration and has pleiotropic activities on immune and inflammatory responses. MIF knock-out mice (MIF = macrophage migration inhibition factor) fail to control Salmonella Typhimurium infection [62] because of a reduced Th1 response i.e. decreased levels of IL-12, IFNγ, and TNFα. Finally, proper functioning of the classical complement pathway is relevant for salmonella infections, as C1q-deficient mice were more susceptible [63].

8. CONFOUNDERS OF GENETIC STUDIES IN HUMANS

A group that deserves special attention in studying susceptibility to salmonella infections is the elderly that are prone to a severe outcome of such infections [64]. Due to immunosenescence, elderly generally show an impaired immune response as compared to younger adults. They show for instance an increased production of proinflammatory cytokines, which is associated with an impaired humoral immune response, but many other responses of the immune function (e.g. vaccination response, T-cell response) seem to have altered during ageing. In addition, the elderly have a higher gastric pH, and are more frequently deficient in micronutrients. It is therefore not surprising, that elderly are more prone to severe salmonella infections. Secondly, infants are relatively susceptible to Salmonella. They still have an immature intestinal microflora, and lack an adequate immune defence to combat pathogens.

Another point of to be remembered is the immune memory. Infections in the past will result in Th1-type immune memory and anti-Salmonella antibodies that prevent or at least confine the clinical outcome of a re-infection with (virulent) Salmonella micro-organisms [6]. Life style factors, like psychological stress, insufficient hygienic measures, anti-microbial treatment (antibiotics) and the high consumption of over the counter (and prescribed) anti-acid drugs to treat gastric ulcers (anti-acida, proton pump inhibitors), will also negatively affect the host resistance to salmonella infections. In addition, unbalanced food consumption including the use of statins may increase infection susceptibility via some immuno-modulatory mechanism. On the other hand, the use of probiotics (Yakult) and previously experienced infections probably protect the host i.e. decrease the host susceptibility to infections.

To address the impact of life style factors and other relevant determinants that affect an adequate functioning of the immune defence, a holistic approach to measure the overall-effect should be performed in high versus low risk groups. The holistic approach includes the measurement of the vaccination response or the delayed type hypersensitivity reaction, or representative parameters of complement system (complement releasing factor; CRF) or macrophage activation (neopterin).

9. CONCLUSIONS

The susceptibility of the host to salmonella infections is partly under control of genetic factors related to both the innate and cellular immune system. Intracellular survival in phagocytes is a typical feature of salmonella, implicating that to fully control salmonella infections the so-called T-helper-1 response (type 1 pathway) is required to adequately combat these pathogens. Various studies, including those performed in humans emphasise the essential role played by type 1 cytokines, like IFN-γ, IL-12 and TNF-α. Aberrations (genetic defect; mutation) in the function of these type 1 pathway cytokines i.e. no proper synthesis of the protein and/or their specific receptors results in increased susceptibility to salmonella infections. Until now, human studies have only been performed on the level of mutations in the type 1 pathway in patient's particular prone to infections with intracellular bacteria.

One should, however, not only focus on the type 1 pathway, as the immune defence system against salmonella also comprises a number of other functions, like passage of the stomach, entry in the sub-epithelium and the innate immune response. Especially rodent studies have indicated that defects in these functions increase the host susceptibility to salmonella infections. Factors or mediators to be mentioned in this respect are e.g. Nramp1, TLR4, LPS-binding protein, CD14, NO-synthase type 2, and NADPH-oxidase.

It is concluded that polymorphisms in both the type 1 cascade and crucial elements of the innate immune response may be associated with an enhanced susceptibility to Salmonella in certain individuals. These factors have not been investigated yet, but may generate relevant additional information on the subject's susceptibility to become infected and ill, even after the exposure to low doses of the pathogen.

Finally, realising that he association between susceptibility to infections and polymorphisms in relevant genes is confounded by many life style related co-variables and previously experienced infections, these variables should receive attention in studying genetic susceptibility.

10. DESIGN OF FUTURE STUDIES

10.1 Rodent studies

Animal models are suitable to determine the physiological relevance of genetic defects for the infection risk of pathogens, including salmonella. In selecting of the model (mouse strain and gene), one should attend the relevancy for humans i.e. are such polymorphisms present in human genes.

Infection of rodents, with *S.* Typhimurium or *S.* Enteritidis induces much more severe symptoms than usually seen in humans. In humans, the infection is typically confined to the gastro-intestinal tract whereas in rodents an invasive illness is usually seen with generalised septicaemia. Although it is difficult to clearly define the differences between these two species with respect to the pathogenesis, it seems that humans and rodents do show a similar innate immune response to Salmonella. In contrast, the specific immune response seems to differ between these two species. The Salmonella-rodent model may be valid for studying the initial stages of infection. It is therefore attractive to study salmonella infections in rodent models with specific defects in the innate immune response. Rodent models that would be of interest for future study of genetic susceptibility for salmonella infections are mice deficient in Nramp1, TLR4 or rodents with some genetic defect in the type 1 pathway. In addition, such studies will provide information on the effect of relative low loadS of Salmonella, because deficient animals are usually more sensitive to these pathogens.

10.2 Human studies

Obviously, the optimal approach is to use prospective studies, but such studies are elaborative and expensive. An alternative is to perform controlled cross-sectional studies in specific subpopulations at risk or between subpopulations that have shown differences in the incidence of salmonella infections. Polymorphisms both in members of the Th1-pathway seem to be promising, and in other factors more or less linked to the innate immune response should be studied (cf. Conclusions). In addition to investigate genetic polymorphisms, it is advocated to determine non-genetic co-variables (confounders), like various life style factors.

An elegant approach is the Campylobacter-Salmonella patient control study where high and low risk groups were formed based on a questionnaire and proven salmonella infection. The feasibility to study DNA-polymorphisms in this cohort is currently investigated.

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ANNEX 1. GENETIC POLYMORPHISMS IN HEALTHY SUBJECTS.

TNF-238 (=-418)

%GG	%GA	%AA	%G	%A	Population	N	Reference
89	11	0	94	6	U.K.	88	[65]
91	4	5	93	7	African USA	74	[66]

TNF-308 (= -488)

59	45	6	74	26	U.K.	556	[67]
57	34	2	80	20	U.K.	88	[65]
70	29	1	84	16	Canada	281	[68]
60	36	4	78	22	Ireland	389	[69]
87	12	1	93	7	African USA	74	[66]

TNF-850 (= -1021)

74	23	3	86	14	African USA	74	[66]

TNF-856 (= -1027)

88	11	1	94	6	African USA	74	[66]

CD14

- Localised on chromosome 5q31.1.
- expressed on monocytes and macrophages, and less on neutrophils, Langerhans cells, dendritic cells, B-cells; not on T-cells or NK cells.
- Polymorphisms found on positions -159, -260, -809, -1145, -1359, -1619, 1344

C-260T

%CC	%CT	%TT	%C	%T	Population	N	Reference
28	50	22	53	47	Ireland	287	[70]
23	51	26	48	52	Ireland	338	[71]
26	47	27	49	51	Italian	215	[72]
35	47	19	57	43	Dutch	58	[73]

C-159T

%CC	%CT	%TT	%T	%C	Population	N	Reference
20	52	28	46	54	Australian	107	[74]
31	49	20	55	45	German	650	[75]
20	54	27	55	54	Dutch	158	[76]
22	61	18	46	54	German children	800	[77]
35	57	8	39	61	American	49	[78]
35	44	21	43	57	Norwegian	117	[79]
27	51	22	52	48	German	444	[80]
34	51	16	59	41	Finnish alcoholics	381	[81]
27	50	23	49	52	USA cauc. children	163	[82]
26	52	22	52	48	Americans	39	[83]

TLR4

- Toll-like receptor-4 for signal transduction from CD14 to cytoplasm.
- expressed on macrophages and dendritic cells
- Polymorphisms:

D259G = Asp299Gly = A896G
T359I = Thr399Ile = C1196T:
A137G in 2.4%;

A2026G	62% A; 38% G	N=116 [84]
T1607C	90% TT; 10% CT	N=116 [84]

Asp299Gly

%AA	%AG	%GG	%A	%G	Population	N	Reference
89	11	0.5	89	11	U.K.	179	[85]
88	11	1.2	93	7	U.S.A.	83	[45]
90	9	1.3	95	5	U.K.	879	[86]
90	10	0.6	94	6	Dutch cardiac pat.	159	[87]
84	16	0	92	8	Belgium caucasian	116	[84]
91	8	0.8	95	5	Belgium caucasian	140	[88]
91	9	0	95	5	German	204	[89]

Thr399Ile

%AA	%AG	%GG	%A	%G	Population	N	Reference
87	13	0	93	7	U.S.A.	39	[83]
90	10	0	95	5	Scotland	80	[90]
89	11.3		94	6	Dutch cardiac pat.	159	[87]
90	10	0	95	5	German	204	[89]

NRAMP1

Soborg (ref. 91): Denmark; Marquet (ref. 92): Colombia

INT4	GG 43%; GC 49%; CC 8%	N=176 [91]
5'(CA)n *	199/199 43%; 199/other 46%; other/other 11%	N=176 [91]
3'UTR	ND/ND 95%; ND/D 5%	N=176 [91]
D543N	GG 94%; GA 6%	N=176 [91]
3'UTR	ND/ND 18%; ND/D 53%; D/D 28%	N=135 [92]
D543N	GG 91%; GA 8%; AA 1%	N=135 [92]
5'GT repeat	286/286 43%; 286/288 48%; 286/286 10%	N=135 [92]
274C/T	CC 41%; CT 50%; TT 10%	N=135 [92]
469G/T	GG 42%; GT 50% TT 8%	N=135 [92]
823C/T	CC 93%; CT 7%; TT 0%	N=135 [92]

* =5'GT repeat

IL-12 en IL-12receptor

Polymorphisms:
IL-12p35: -1250 T/A, -666 T/G
IL-12p40: -5230 A/G, -5251 C/T, -3882 A/G, -5310 T/A

In african USA:

IL-12p35	-1250 T/A	TT 100%	N=74 [66]
IL-12p35	-666 T/G	TT 80%; TG 19%; GG 1%	N=74 [66]

IL12p40 gene 1188 in 3'UTR

%AA	%AC	%CC	%A	%C	Population	N	Reference
61	32	7	77	23	U.K. caucasian	157	[93]
68	26	6	81	19	USA mixed	145	[94]
52	41	7	72	28	Italian	140	[95]

In African USA N=74

IL-12p40	-5230 A/G	AA 100%	[66]
IL-12p40	-5251 C/T	CC 99%; CT 1%	[66]
IL-12p40	-3882 A/G	AA 100%	[66]
IL-12p40	-5310 TA	TT 97%; TA 3%	[66]

IL12Rbeta1 705 A/G AA 70%; AB 18%; BB 12% N=33 Japan [96]

IFN-g

5644 3'UTR

%AA	%AG	%GG	%A	%G	Population	N	Reference
36	49	15	61	39	U.K. caucasian	157	[93]
30	47	23	53	47	Italian	140	[95]

874 T/A TT 21%; TA 48%; AA 31% U.K. N=222 [97]

IL-6

174 G/C (=pos. 236)

%GG	%GC	%CC	%A	%C	Population	N	Reference
32	51	15	59	41	Ireland	389	[69]
40	46	14	63	37	African USA	74	[66]
35	45	20	57	43	U.K.	224	[97]
41	50	9	66	34	Italian	140	[95]

ANNEX 2. STUDY APPROACHES

1. CaSa-study design

Participants from a recent Dutch case-control study (CaSa-study) form the subjects of this study also. This case-control study of risk factors for human salmonellosis was carried out in the Netherlands in 2002-2003. Cases were laboratory-confirmed patients with salmonella infection. Controls were selected from the population registries of 25 municipalities by prospective frequency matching for age, sex, degree of urbanisation and season. In our study cases and controls will (at a minimum) be matched for age and sex.

Sample size selection:

From previous studies and expert opinion, it is estimated that 10% of the population will have the polymorphism of interest and that this increases to approximately 14% in cases. We would be interested in the conventional alpha level of 0.05 and beta level of 0.20 representing a power of 80% to detect an effect of this magnitude if it truly exists.

Sample size calculation using above values (calculated with epi-info statcalc).

Confidence interval	Power	cases:control (ratio)	Odds ratio	Cases (n)	Controls (n)
95%	80%	1:1	1.4	1413	1413
95%	80%	1:2	1.4	1041	2082
95%	80%	1:3	1.4	917	2751
95%	80%	1:4	1.4	855	3420
95%	80%	1:1	1.8	436	436
95%	80%	1:1	2.0	219	219

From the cases control study we have in total 573 laboratory confirmed cases and 3409 controls. By taking a higher number of controls to cases (for example 4 cases per control) we can reduce the number of cases required but even if all agreed to consent we would have insufficient number (573 versus 855). However, the greatest benefit in these types of genetic studies is in taking a maximum of two controls per case.

2. Outbreak approach

OSIRIS (internet based system) provides data on outbreaks of foodbourne infections. This information is from the GGD. However the number available from this source to small.

Year	Species	Total patients	Outbreaks
2003	S.Enteridis	85	14
	S.Typhimurium	0	0
2002	S.Enteridis	48	9
	S.Typhimurium	0	0
2001	S.Enteridis	104	13
	S.Typhimurium	2	1

ANNEX 3. SAMPLE SIZE CALCULATION

Calculation 1.

Ratio case: control	Prevalence polymorphism (controls)	sample size estimate case: controls		sample size 100% respond	60% respond
1:1	10%	1413	1413	2826	4710
1:2		917	2751	3123	5205
1:1	15%	1015	1015	2030	3383
1:2		750	1500	2250	3750
1:1	20%	823	823	1646	2743
1:2		610	1220	1830	3050
1:1	25%	715	715	1430	2383
1:2		531	1062	1593	2655
1:1	30%	649	649	1298	2163
1:2		483	966	1449	2415
1:1	35%	610	610	1220	2033
1:2		455	910	1365	2275
1:1	40%	588	588	1176	1960
1:2		440	880	1320	2200
1:1	45%	580	583	1163	1938
1:2		434	868	1302	2170
1:1	50%	584	584	1168	1946
1:2		438	876	1314	2190

Sample size calculation on basis of:

- odds ratio 1.4,
- Power 80%,
- 95% confidence interval
- various background prevalence of polymorphism

Calculation 2.

Ratio case: control	Prevalence polymorphism (controls)	sample size estimate case: controls		sample size (100% respond)	60% (respond)
1:1	10%	701	701	1402	2337
1:2		514	1028	1542	2570
1:1	15%	508	508	1016	1693
1:2		373	746	1119	1865
1:1	20%	415	415	830	1383
1:2		306	612	918	1530
1:1	25%	363	363	726	1210
1:2		269	538	807	1345
1:1	30%	332	332	664	1107
1:2		247	494	741	1235
1:1	35%	314	314	628	1047
1:2		234	468	702	1170
1:1	40%	305	305	610	1017
1:2		228	456	684	1140
1:1	45%	303	303	606	1010
1:2		227	454	1302	731
1:1	50%	307	307	614	1023
1:2		231	462	693	1155

Sample size calculation on basis of:

- odds ratio 1.6,
- Power 80%,
- 95% confidence interval
- various background prevalence of polymorphism