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Diagnostic real-time PCR for detection of *Salmonella* in food and characterization of epidemiologically important *Salmonella enterica* subsp. *enterica* serovars isolated from livestock, food and humans

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CONTENT

PREFACE

This habilitation thesis consists of 11 peer-reviewed publications related to three subject areas:

1. Development and validation of diagnostic real-time PCR for detection of *Salmonella* in food

   (Publications 1-3)

2. Enumeration of *Salmonella* bacteria in food by real-time PCR

   (Publications 4-5)

3. Characterization of epidemiologically important *Salmonella enterica* subsp. *enterica* serovars isolated from livestock, food and humans

   (Publications 6-11)

Initially, an introduction and literature survey are given, followed by the aim of thesis as well as a summary of methods and results for each subject area. A cross-sectional discussion from all 11 publications including highlights of major future trends in molecular diagnosis and surveillance in food microbiology follows.

Publications part of this habilitation thesis

Publication 1

Malorny, B., Paccassoni, E., Fach, P., Bunge, C., Martin, A. and Helmuth, R. 2004. Diagnostic real-time PCR for detection of *Salmonella* in food. Appl Environ Microbiol 70, 7046-7052.

Publication 2

Malorny, B., Mäde, D., Teufel, P., Berghof-Jäger, C., Huber, I., Anderson, A. and Helmuth, R. 2007. Multicenter validation study of two blockcycler- and one capillary-based real-time PCR methods for the detection of *Salmonella* in milk powder. Int J Food Microbiol 117, 211-218.

Publication 3

Malorny, B., Bunge, C. and Helmuth, R. 2007. A real-time PCR for the detection of *Salmonella* Enteritidis in poultry meat and consumption eggs. J Microbiol Methods 70, 245-251.

Publication 4

Malorny, B., Löfström, C., Wagner, M., Krämer, N. and Hoorfar, J. 2008. Enumeration of *Salmonella* bacteria in food and feed samples by real-time PCR for quantitative microbial risk assessment. Appl Environ Microbiol 74, 1299-1304.

Publication 5

Krämer, N., Löfström, C., Vigre, H., Hoorfar, J., Bunge, C. and Malorny, B. 2011. A novel strategy to obtain quantitative data for modelling: combined enrichment and real-time PCR for enumeration of salmonellae from pig carcasses. Int J Food Microbiol 145 Suppl 1, S86-S95.

Publication 6

Huehn, S., Bunge, C., Junker, E., Helmuth, R. and Malorny, B. 2009. Poultry-associated *Salmonella enterica* subsp. *enterica* serovar 4,12:d:- reveals high clonality and a distinct pathogenicity gene repertoire. Appl Environ Microbiol 75, 1011-1020.

Publication 7

Huehn, S., Helmuth, R., Bunge, C., Guerra, B., Junker, E., Davies, R.H., Wattiau, P., van Pelt, W. and Malorny, B. 2009. Characterization of pathogenic and resistant genome repertoire reveals two clonal lines in *Salmonella enterica* subsp. *enterica* serovar Paratyphi B (+)-tartrate positive. Foodborne Pathog Dis 6, 431-443.

Publication 8

Huehn, S., La Ragione, R.M., Anjum, M., Saunders, M., Woodward, M.J., Bunge, C., Helmuth, R., Hauser, E., Guerra, B., Beutlich, J., Brisabois, A., Peters, T., Svensson, L., Madajczak, G., Litrup, E., Imre, A., Herrera-Leon, S., Mevius, D., Newell, D.G. and Malorny, B. 2010. Virulotyping and antimicrobial resistance typing of *Salmonella enterica* serovars relevant to human health in Europe. Foodborne Pathog Dis 7, 523-535.

Publication 9

Hauser, E., Tietze, E., Helmuth, R., Junker, E., Blank, K., Prager, R., Rabsch, W., Appel, B., Fruth, A. and Malorny, B. 2010. Pork contaminated with *Salmonella enterica* serovar 4,[5],12:i:-, an emerging health risk for humans. Appl Environ Microbiol 76, 4601-4610.

Publication 10

Hauser, E., Hebner, F., Tietze, E., Helmuth, R., Junker, E., Prager, R., Schroeter, A., Rabsch, W., Fruth, A. and Malorny, B. 2011. Diversity of *Salmonella enterica* serovar Derby isolated from pig, pork and humans in Germany. Int J Food Microbiol 151, 141-149.

Publication 11

Hauser, E., Tietze, E., Helmuth, R., Junker, E., Prager, R., Schroeter, A., Rabsch, W., Fruth, A., Toboldt, A. and Malorny, B. 2012. Clonal dissemination of *Salmonella enterica* serovar Infantis in Germany. Foodborne Pathog Dis 9, 352-360.

1. Introduction and literature survey

Salmonella enterica is a bacterial food-borne pathogen that causes gastroenteritis and occasionally systemic infections in humans and animals. It is estimated that *Salmonella* is responsible for approximately 93.8 million human cases, with 155,000 deaths annually worldwide (Majowicz et al., 2010). The real number of infections is probably significantly higher due to misdiagnosis and underreporting of gastrointestinal illnesses (Voetsch et al., 2004). Because the medical and economic burden of diarrheal diseases caused by *Salmonella* species in developing and developed countries is considerable there is worldwide interest in lowering *Salmonella* infections. In Europe, a number of *Salmonella* control programmes, primarily initiated for poultry and poultry products, have been approved with the aim of decreasing the number of human infections caused by *Salmonella* (Anonymous, 2003). Specific European Union (EU)-wide regulations were passed for the reduction of the prevalence of certain *Salmonella* serovars in breeding flocks of *Gallus gallus* (Anonymous, 2005a), in laying hens of *Gallus gallus* (Anonymous, 2006; Anonymous, 2011a) and in turkeys (Anonymous, 2008; Anonymous, 2012). Furthermore table eggs from infected flocks of laying hens, as one most important vehicle for infection, are strongly restricted in their placement on the market (Anonymous, 2007a).

The diagnosis, surveillance and control of *Salmonella* require tools for sensitive and rapid detection and characterization of the pathogen in livestock and food production. The identification of the various sources of *Salmonella* and the characterization of their subgroups will contribute to preventing subsequent human exposure. Usually, culture-based microbiological testing of foods is accepted and is applied as an integral part of the global food production and food safety regulations. Indeed, it remains a critically important method in terms of rapidity, sensitivity and resolution. Genotypic-based methods including the characterization of the pathogenic potential (referred to also as virulotyping) of a *Salmonella* subgroup may prove to be an important pillar to more closely meet the expectations of the food industry and legislation (Hoorfar et al., 2011a). The acceptance of alternative methods in legislation for food control and for the investigation of foodborne outbreaks depends substantially on the validity and standardization approach. Considerable work has been carried out that culminated in the publication of an ISO Standard 16140:2003 (ISO, 2003a), a protocol for the validation of alternative test methods based on internationally accepted criteria. Recently, a new Standard (ISO 22118:2011) specified the minimal requirements of performance characteristics for the detection and quantification of

nucleic acids by molecular methods (ISO, 2011a). However, currently the validation process is only applicable for qualitative or quantitative detection methods but not for genotypic methods.

This thesis contributes to providing, on the one hand, validated methods for the qualitative and quantitative detection of *Salmonella* in food and, on the other hand, studies on epidemiologically important *S. enterica* serovars in order to describe and to estimate their potential hazard for humans in relation to the food chain production. In the following the taxonomy, epidemiology, food aspects and analytical methods for the detection and characterization of *Salmonella* are summarized.

1.1 Taxonomy and traditional schemes for *Salmonella* typing

The genus *Salmonella* is a member of the *Enterobacteriaceae* family composed of rod-shaped, facultative anaerobic, catalase-positive, oxidase-negative, motile and gram-negative bacteria (Farmer III, 2003). The genus was officially named after the American pathologist Daniel Elmer Salmon. His colleague, T. Smith, first isolated the bacterium from porcine intestine in 1884 as a common cause of hog cholera and designated the type-strain *Bacillus cholerae suis* (Smith, 1894). Later the name was changed to *Salmonella cholerae-suis* by J. Lignières in 1900. The nomenclature of *Salmonella* has undergone many changes within the past decades (Euzéby, 1999; Brenner et al., 2000; Tindall et al., 2005). Currently it is accepted that the genus consists of two species: *Salmonella enterica* and *Salmonella bongori* (Reeves et al., 1989). The species *S. enterica* is subdivided into six subspecies (subsp.): *S. enterica* subsp. *enterica* (designated subspecies I), *S. enterica* subsp. *salamae* (subspecies II), *S. enterica* subsp. *arizonae* (subspecies IIIa), *S. enterica* subsp. *diarizonae* (subspecies IIIb), *S. enterica* subsp. *houtenae* (subspecies IV) and *S. enterica* subsp. *indica* (subspecies VI). Species and subspecies can be distinguished on the basis of differential biochemical properties (Grimont and Weil, 2007) or electrophoretic types (ETs) based on multilocus enzyme electrophoresis (MLEE) (Reeves et al., 1989). Meanwhile the phylogenetic relationship of *Salmonella* species and subspecies was confirmed by other methods, e.g. DNA microarrays (Porwollik et al., 2002) or MALDI-TOF (Dieckman et al., 2008).

According to the White-Kauffmann-Le Minor scheme (Grimont and Weil, 2007), *Salmonella* bacteria can be classified into serovars (serotypes) based on their reactivity to monovalent antisera. Since its introduction by White in 1934 (International Salmonella

Subcommittee, 1934) the subtyping scheme has distinguished 46 lipopolysaccharide moieties (O-antigens) and 114 different flagellar antigens (H-antigens), as well as for some serovars the capsular polysaccharide (Vi-antigen). A single *Salmonella* cell can express two different H-antigens but only one at a time. The expression of the two flagellin loci is regulated by a phase variation mechanism by the invertible element *hin* (Silverman and Simon, 1980). The acquisition of the genetic elements for flagellar phase variation was a major transition in the evolution of *Salmonell*a, possibly a key step in the expansion of *Salmonella* as a pathogen for warm-blooded hosts (Li et al., 1995) because *S. enterica* subsp. I, II, IIIb and VI are diphasic strains of *Salmonella* which have been found to cause disease in warm-blooded organisms. In contrast, *S. enterica* subsp. IIIa, IV and *S. bongori* are characteristically lacking one flagellar phase and usually originate from cold-blooded animals and the environment (McQuiston et al., 2008). Occasionally serovars belonging to diphasic *S. enterica* subspecies have again lost the second flagellum locus, possibly an adaptation process in preferential or restricted habitats (McQuiston et al., 2008). The serotyping scheme is used worldwide as an epidemiological and standardized typing method for *Salmonella*. Currently, 2610 serovars are known, of which 99% belong to *S. enterica* and 59% to *S. enterica* subsp. *enterica*.

A serovar is designated through a unique combination of the O-, H1- and H2-antigens (antigenic formula) separated by colons, respectively. Before 1966 all serovars of all subspecies with the exception of subspecies IIIa and IIIb were usually named after the first geographic location of isolation. Some serovar names express the disease or relationship, or are correlated with disease and host specificity (Grimont and Weil, 2007). Afterwards, only strains belonging to *S. enterica* subsp. *enterica* were named and previous names in subspecies II, IV and VI and *S. bongori* were again erased. For example, the antigenic formula of serovar 1,4,[5],12:i:1,2 is assigned to the *S. enterica* subsp. *enterica* serovar Typhimurium.

For the discrimination of strains belonging to the same serovar, phage typing is traditionally performed. It is useful in outbreak and epidemiological investigations. The method is based on the susceptibility of strains to a panel of bacteriophages. Prominent phage type systems are available for *S. enterica* serovars Typhimurium (Anderson et al., 1977) and Enteritidis (Ward et al., 1987). The current phage typing scheme for *S. enterica* serovar Typhimurium recognizes 207 definitive and many other provisional phage types

(Rabsch, 2007). For *S. enterica* serovar Enteritidis 87 phage types are currently defined (A. Schroeter, personal communication).

1.2 Host range of *S. enterica* serovars

Salmonella serovars can be subdivided into three groups on the basis of host prevalence and pathogenic hazard (Wallis and Barrow, 2005). They can differ substantially in clinical manifestations, ranging from an asymptomatic state to severe illness (Jones et al., 2008). Serovars of the first group are known to be highly host-adapted, causing systemic disease in a limited number of related species. The most prominent representative is *S. enterica* serovar Typhi, which causes typhoid disease only in humans and some non-human primates. *S. enterica* serovar Gallinarum, is predominantly an avian-adapted serovar, the causative agent of fowl typhoid. The second group consists of host-restricted serovars that cause systemic disease in specific animals but may also rarely infect other mammals (Kingsley and Bäumler, 2000). For example, *S. enterica* serovar Choleraesuis causes systemic paratyphoid illness in pigs but infrequently infects humans. Similarly, *S. enterica* serovar Dublin is usually restricted to cattle, causing systemic disease, but invasive human infections are occasionally reported (Wollin, 2007) and it is capable of causing typhoid fever-like infections in mice (Barrow et al., 1994). In contrast, the third group can infect a broad range of avian and mammalian hosts with a wide range of diseases. The most prominent serovars of this group are *S. enterica* serovars Typhimurium and Enteritidis. In newly hatched chicks *S. enterica* serovars Enteritidis and Typhimurium cause systemic disease and gastroenteritis whereas older chickens are asymptomatic carriers. In calves, *S. enterica* serovar Typhimurium causes enterocolitis including dehydration. In mice, *S. enterica* serovar Typhimurium causes typhoid fever-like disease (Tsolis et al., 1999). Immuno-competent humans often suffer from self-limiting diarrhoea but immuno-compromised individuals can develop systemic disease with high mortality rates (Kingsley et al., 2009; Dougan et al., 2011). Variants of *S. enterica* serovar Typhimurium are associated with specific avian paratyphoid disease in pigeons and other birds and these may be considered host-adapted (Rabsch et al., 2002). A genetic understanding of virulence, host adaptation and host specificity is still poor. Host adaptation can be triggered by the specific organization of the immune system in birds, mammals or cold-blooded vertebrates leading to an adapted pathogenicity gene repertoire of the serovar (Bäumler et al., 1998; Kingsley and Bäumler, 2002). The increasing number of available pathogen and

host genome sequences combined with specific animal models will doubtless result in new approaches in that field in the future (Suar et al., 2006; Dougan et al., 2011).

1.3 Epidemiology of *Salmonella*

Salmonellae occur worldwide in humans, animals and the environment but with different frequencies. They are zoonotic with the ability to be transferred from animals to humans or vice versa. The natural habitat is usually the intestine. The route of infection from animals to humans is mostly through contaminated food and the primary sources are food of animal origin especially poultry, pigs and cattle (Thorns, 2000). Other *Salmonella* infections can be caused by human-to-human transmission, waterborne transmission or environmental or animal exposure (Bertrand et al., 2008). Pets, especially reptiles, often act as a reservoir for *Salmonella* and are responsible for infections due to direct or indirect contact with these animals (Bertrand et al., 2008).

Salmonella infections have been increasingly linked to the consumption of vegetables and fruit (Lynch et al., 2009). Produce can be contaminated with *Salmonella* in the field through the application of manure, fertilizers, pesticides and irrigation with contaminated water. Insects and animals may be another source of *Salmonella* entry. Post-harvest cross-contamination can occur during washing, packaging and transport (Beuchat, 2002). Sprout seeds, in particular, have been the cause of larger outbreaks (CDC, 2009; Rimhanne-Finne et al., 2011; Rosner et al., 2012).

The infection dose of *Salmonella* to induce illness in human depends on several factors, such as immunological status and age of the host, the food vehicle and strain properties (Blaser and Newman, 1982). Generally, studies showed that the higher the dose, the higher the probability of becoming ill. Studies on 116 volunteers showed that the lowest dose causing illness was 1 x 10^5 *S. enterica* serovar Typhi organisms with 28% attack rate using milk as the vehicle (Hornick et al., 1970). However, data from outbreaks often showed that a considerably lower number of ingested organisms caused illness (D'Aoust and Pivnick, 1976). Especially fatty vehicles (chocolate, cheese) may protect salmonellae from the bactericidal action of gastric acidity (D'Aoust, 1994).

1.3.1 Incidence of human salmonellosis and outbreaks

S. enterica subsp. *enterica* accounts for approximately 99% of *Salmonella* infections in humans and warm-blooded animals (Farmer III, 2003). In Germany, since the mid-1990s the reported number of confirmed cases has steadily continued to decrease, with the

exception of 2006 and 2007. For 2010, 25,307 human *Salmonella* infections were reported, a decrease of 19% compared to the 2009 data (RKI, 2011). Twenty-six patients died of salmonellosis. The incidence notification rate in Germany was 30.9 cases per 100,000 population and significantly lower than the average of the last five years (median 63.4 cases per population). A significant part of the decrease is explained by declining rates of *S. enterica* serovar Enteritidis infections since 2001 (Frank et al., 2009). However, in 2010 47% of all infections were still caused by *S. enterica* serovar Enteritidis (2009: 58%). The second most common serovar is *S. enterica* serovar Typhimurium (including monophasic Typhimurium), with 41% (2009: 33%). The relative increase is the consequence of decreasing *S. enterica* serovar Enteritidis cases as well as the rising number of infections caused by the monophasic variant of *S. enterica* serovar Typhimurium (4,[5],12:i:-). Since 1999 the National Reference Centre for Salmonella and other Enterics serotyped steadily increasing numbers of isolates from humans belonging to the monophasic variant *S. enterica* serovar Typhimurium (0.1% in 1999 to 24.9% in 2011) (Erhard Tietze, personal communication). Further serovars causing human salmonellosis are *S. enterica* serovar Infantis (2%), Derby (0.8%), Kentucky and Virchow (0.5%). All other serovars caused 8.5% of all cases.

An outbreak is defined as either a household outbreak, where only members of a single household are affected, or as a general outbreak, where members of more than one household are affected (EFSA and ECDC, 2011). In 2010 for Germany, altogether 562 outbreaks with 2,108 cases were reported (RKI, 2011). In three outbreaks more than 40 humans were affected. The largest outbreak involved 110 persons, especially children, and was caused by *S. enterica* serovar Enteritidis (RKI, 2011). Two kindergartens were delivered with meals from the same caterer. The source of the infections could be not identified. Recently, another large outbreak affected 106 individuals in October/November 2011. The outbreak strain belonged to *S. enterica* serovar Newport. The vehicle of infection was mungbean sprouts imported from the Netherlands (Rosner et al., 2012).

In Europe, in 2010, 99,020 salmonellosis cases were reported. A 5-year trend (2006-2010) shows a statistically significant decrease in the case numbers (EFSA and ECDC, 2012). The incidence notification rate was on average 21.5 cases per 100,000 population compared to 23.7 cases per population in 2009 ranging from 1.9 in Portugal to 91.1 confirmed cases per 100,000 population in Slovakia. Sixty-two humans died due to non-typhoidal salmonellosis among 46,639 confirmed *Salmonella* cases. The two most

commonly reported serovars were *S. enterica* serovar Enteritidis and *S. enterica* serovar Typhimurium, representing 45.0% and 22.4% of all reported serovars in confirmed human cases. A decrease was recognized for both serovars in comparison to 2009 (7.3% and 0.9%, respectively). Further *S. enterica* serovars were Infantis (1.8%), monophasic Typhimurium (seroformula 4,[5],12:i:-) (1.5%), Newport (0.9%), Kentucky (0.8%), Virchow and Derby (each 0.7%), Mbandaka and Agona (each 0.5%). Other serovars covered 25.3% of *Salmonella* infections in Europe (EFSA and ECDC, 2012).

According to the salmonellosis cases, *Salmonella* outbreaks within the EU in the years 2007 to 2010 declined sharply from 2,253 to 1,604 outbreaks (verified and possible outbreaks). In 2010, the predominant serovar involved in outbreaks was *S. enterica* serovar Enteritidis (61.3%). Eggs and egg products were the cause in 43.7% of all strong evidence *Salmonella* outbreaks. Inadequately heat-treated bakery products using raw eggs were the second most frequently known source of *Salmonella* infections (14.4% of verified outbreaks) (EFSA and ECDC, 2012).

Similarly, reported data collected between 2001 and 2007 from the World Health Organization Global Foodborne Infections Network including 37 countries showed that *S. enterica* serovars Enteritidis (43.5%) and Typhimurium (17.1%) were the most common serovars isolated from humans worldwide (Hendriksen et al., 2011). In developing countries, the proportion of *S. enterica* serovar Enteritidis decreased from 73.9% in 2001 to 55% in 2007 and in developed countries the proportion of *S. enterica* serovar Typhimurium decreased from 26.4% to 18.8%. *S. enterica* serovars Newport (3.5%), Infantis (1.8%), Virchow (1.5%), Hadar (1.5%) and Agona (0.8%) were also frequently isolated (Hendriksen et al., 2011). However, regional differences in prevalence of *Salmonella* serovars have been observed. For example, *S. enterica* serovar Heidelberg was much more frequently reported from North America (top 4) than from Europe (top 9) and Latin America (top 19) and did not occur in the African or Asian region among the 20 most common serovars.

1.3.2 Incidence of *Salmonella* in livestock

Often, animals are asymptomatic carriers of *Salmonella* and carry the bacteria in the intestine. They can shed relatively large numbers of salmonellae in the faeces over a long period. Consequently, the control of *Salmonella* in food producing animals is imperative to avoid the transmission along the food chain to humans. Usually, in developed countries livestock is regularly monitored in order to estimate the prevalence of the pathogen and

serovars and to perform control measures. Livestock can be infected with *Salmonella* by vertical transmission through infected parents or by horizontal transmission between animals, faeces, feed, or vehicles such as rodents or birds (Davies et al., 2004; Vandeplas et al., 2010).

The incidence of *Salmonella* in livestock depends on several factors such as the conditions for intensive animal husbandry, hygiene measures and climate conditions. The incidence of *Salmonella* in Northern countries is often lower than in those located in warmer climate zones. Furthermore, *Salmonella* cases are subject to a seasonal variation with higher rates in the summer and autumn, and a decline in the winter months (EFSA and ECDC, 2011). Contaminated animal feed and wild animals (e.g. birds) have been recognized as important entry sites into the food chain in farm livestock (Skov et al., 2008). Another source of contamination is the slaughter process of the animals (Bolton et al., 2003).

Poultry can be infected by many different serovars. The avian-adapted *S. enterica* serovars Gallinarum and Pullorum causing severe systemic disease (fowl typhoid) in birds were formerly highly prevalent in chickens and still cause serious economic problems in many countries with low hygiene standards (Barrow and Freitas Neto, 2011). Vaccination programmes in the middle of the twentieth century largely eradicated the serovars from Europe and North America (Barrow et al., 2012). Since the mid-1980s *S. enterica* serovar Enteritidis has established itself as the most frequently isolated serovar from poultry, especially chickens, in many parts of the world possibly by filling the ecological niche vacated by *S. enterica* serovars Gallinarum and Pullorum (Bäumler et al., 2000; Ward et al., 2000). In consequence, *S. enterica* serovar Enteritidis is attaining major public health significance because it is dominantly infecting humans, transmitted by food products (EFSA and ECDC, 2012). Despite vaccination programmes it is highly prevalent in many parts of the world (Hendriksen et al., 2011). In Europe, baseline surveys on the prevalence of *Salmonella* showed that 30.7% of commercial large-scale laying hen holdings and 23.7% of the broiler flocks of *Gallus gallus* were *Salmonella* positive (EFSA, 2007a; EFSA, 2007b). Most frequently isolated was *S. enterica* serovar Enteritidis followed by *S. enterica* serovar Infantis (Table 1). However, the variation in prevalence and predominant serovars was very large between European countries. For example, the prevalence of *S. enterica* serovar Enteritidis in laying hen holdings ranged from 0% to 79.5%. In Germany, the prevalence was usually slightly below the average of all EU Member States (Table 1). Predominant serovars except for those in laying hen holdings in Germany differed from the

European average. In broiler flocks and carcasses a monophasic serogroup B serovar with seroformula 4,12:d:- was most frequently isolated with a prevalence of 30.7% and 27.6%, respectively (Table 1). The serovar was also isolated in Denmark and the United Kingdom with prevalence of 15.2% and 2.8%, respectively. Other countries were not affected. In turkey a baseline survey performed in 2006/2007 showed 13.6% *Salmonella* positive breeding flocks and 30.7% positive fattening flocks in Europe. Germany had a significantly lower prevalence in fattening flocks (9.2%). Follow-up national zoonosis monitoring programmes within the framework of directive 2003/99/EC (Anonymous, 2003) showed in Germany an ongoing decrease of the *Salmonella* prevalence in laying hens, broilers and turkeys with 6.1%, 6.8% and 2.0% in dust samples and 3.6%, 7.4% and 1.1% in faeces samples, respectively (BVL, 2012).

In pigs, the host-restricted serovar Choleraesuis was predominant in the 1950s and 1960s in Europe (Sojka et al., 1977). After that it decreased dramatically and is today very rarely isolated in Europe. In the United States the serovar is still among the top three of clinical cases in pigs (Foley et al., 2008). Meanwhile *S. enterica* serovar Typhimurium is one of the most common serovars isolated from pigs, in both Europe and the United States (EFSA and ECDC, 2012; Foley et al., 2008). Formerly, a study investigating 11,942 slaughter pigs in Germany in 1996 estimated the overall prevalence of *Salmonella* to be 6.2% (Käsbohrer et al., 2000). Seventy-two percent of the isolates belonged to the *S. enterica* serovar Typhimurium. Two European baseline studies in 2006/2007 and 2008 showed that the serovar is most commonly isolated in slaughter pigs and secondly most commonly in breeding pigs (EFSA, 2008a; EFSA, 2009) (Table 1). The overall *Salmonella* prevalence was significantly higher in breeding pigs than in slaughter pigs (Table 1). In Germany, the second most frequent serovar in slaughter pigs was the monophasic *S. enterica* serovar with the seroformula 4,[5],12:i:-, which was formerly not frequently isolated in pigs. The monophasic serovar started to emerge in Europe in the mid-1990s beginning from Spain (Echeita et al., 1999). Also in the United States it has been recognized as an emerging serovar (Foley et al., 2008). The third predominant serovar in pigs is *S. enterica* serovar Derby. In slaughter pigs it ranks in the top two in Europe and top three in Germany (EFSA, 2008a) (Table 1). Similarly, the serovar was most frequently isolated in non-clinical cases and secondly most frequently isolated in clinical cases of pigs in the United States (Foley et al., 2008).

TABLE 1. Prevalence of *Salmonella* in livestock: Results of baseline surveys.

	European Union, total		Germany	
	% Prevalence	Predominant serovars (%)	% Prevalence	Predominant serovars (%)
Laying hen holdings 2004	30.8	Enteritidis (50.8) Infantis (8.3) Typhimurium (5.2)	28.9	Enteritidis (78.8) Serogroup B rough (30.0) Typhimurium (6.9)
Broiler flocks 2005	23.7	Enteritidis (37.4) Infantis (20.4) Mbandaka (7.9)	15.0	4,12:d:- (30.7) Anatum (20.0) Paratyphi B dT+ (10.7)
Broiler carcasses 2008	15.7	Infantis (29.2) Enteritidis (13.6) Kentucky (6.2)	14.5	4,12:d:- (27.6) Typhimurium (26.3) Paratyphi B dT+ (10.8)
Turkey breeding flocks 2006	13.6	Saintpaul (42.5) Kottbus (17.5) Typhimurium (10.0)	0	none
Turkey fattening flocks 2006	30.7	Bredeney (17.2) Hadar (14.0) Derby (11.3)	9.2	Typhimurium (25.8) Saintpaul (16.1) Hadar (12.9)
Slaughter pigs (lymph nodes) 2006	10.3	Typhimurium (40.0) Derby (14.6) Rissen (5.8)	10.9	Typhimurium (55.0) 4,[5],12:i:- (19.7) Derby (8.9)
Breeding pigs (lymph nodes) 2008	31.8	Derby (29.6) Typhimurium (25.4) Infantis (7.7)	20.6	Derby (40.6) Typhimurium (15.6) Livingstone (9.4)

In cattle, the prevalence of *Salmonella* is generally lower then in poultry or pigs. Monitoring programmes in 10 EU Member States and Norway showed a rate of 0.9% on animal level (EFSA and ECDC, 2012). Bovine salmonellosis is associated primarily with *S. enterica* serovars Dublin and Typhimurium (Wallis and Barrow, 2005). In Germany, between 1995 and 2003 the number of annually registered outbreaks of salmonellosis in cattle was on average 221 ± 26.5 (Methner, 2005). Approximately 50% were caused by *S. enterica* serovar Typhimurium and 15-20% by the cattle-restricted *S. enterica* serovar Dublin.

1.3.3 Incidence of *Salmonella* in food

In the EU and many other countries, eggs and egg products are the foods most frequently implicated in human salmonellosis (Hald et al., 2004; EFSA, 2012). In 2010 13 EU Member States reported *Salmonella* findings in fresh eggs, raw egg at processing, and at retail to be in average 0.4% in batch samples and 0.1% in single samples (EFSA and ECDC, 2012). The highest rates were reported from Ireland (6.1%) and Spain (5.3%). Since 2007, when the *Salmonella* control programmes for flocks of laying hens and broilers were implemented, this represents a reduction by half. *S. enterica* serovar Enteritidis was almost always isolated from table eggs. In Germany, an active monitoring programme on the prevalence in table eggs in 2010 showed that egg yolk samples were not contaminated at all with *Salmonella* but egg bowls with 0.7% (BVL, 2012).

Salmonella prevalence in fresh broiler meat at different levels of production was 4.8% on average in the EU in 2010. This was a decline compared with 2009, but at the same level as in 2008. *S. enterica* serovar Infantis was, at 58.9%, the most frequently isolated serovar followed by *S. enterica* serovar Kentucky (5.7%) and *S. enterica* serovar Paratyphi B dT+ (4.6%) (EFSA and ECDC, 2012). In Germany, a national monitoring programme on the prevalence in fresh broiler meat was conducted in 2009 (BfR, 2011). Rates on the processing level and on retail were 7.4% and 7.6%, therefore differing only slightly. The top three serovars were *S. enterica* serovar Paratyphi B dT+ (37.4%), Infantis (18.6%) and Typhimurium (16.3%) (BfR, 2011). The same study found that the prevalence of *Salmonella* in meat from turkey was, at 5.6%, lower. The dominating serovars were *S. enterica* serovar Saintpaul, 4,[5],12:i:- and Newport, each with 16.6%.

Pork has been identified as another important source for human salmonellosis (EFSA, 2008b; Pires et al., 2012). In Europe the rate of *Salmonella* positive tested samples in fresh pig meat at the production line in 2010 was 0.9%, ranging between 0.3% and 8.9% (EFSA and ECDC, 2012). At processing and cutting plants, *Salmonella* was found in up to 10.4% of fresh pig meat samples and at retail up to 18.5% was reported. The average at retail was 1.0% compared to 0.7% in 2009. In pig meat the dominating serovars in 2010 were *S. enterica* serovar Typhimurium (30.7%), Derby (16.6%) and 4,[5],12:i:- (7.4%). The serovar distribution is similar to that in pigs at primary production, reflecting the transmission of the serovars to humans by pork.

For Germany, the prevalence in pig meat in 2010 was at slaughterhouses 1.0%, and at processing and cutting plants as well as at retail 2% (EFSA and ECDC, 2012). In 2009, as

part of another national monitoring programme in pork, minced meat was found to be positive in 5.3% of the test samples and fresh meat on retail or at processing plant was contaminated in 1.4% and 1.3%, respectively (BfR, 2011). The most frequently isolated serovar was *S. enterica* serovar Typhimurium (50%), followed by *S. enterica* serovars 4,[5],12:i:- (22.2%), Derby and Brandenburg (each 11.1%).

Salmonella in bovine meat is rarely detected in Europe. The average prevalence in 2010 and 2009 was 0.2% (data of seven Member States). *S. enterica* serovars Typhimurium (20.8%), Dublin (18.1%) and monophasic Typhimurium (10%) were the serovars most frequently isolated (EFSA and ECDC, 2012). In Germany, in 2010 the rate of *Salmonella* positive tested bovine meat samples was at retail 0.6% and at processing plant 0.5%. The national monitoring programme in 2009 found that 0.5% of fresh bovine meat and none of the samples at processing plant were contaminated (BVL, 2010). Dominantly isolated *S. enterica* serovars were monophasic Typhimurium (4,[5],12:i:-), Typhimurium and Dublin.

1.4 Diagnostic methods for detection and enumeration of *Salmonella* in food

1.4.1 Traditional culture methods for detection and isolation

For the detection and isolation of *Salmonella* in food and feeding stuff an internationally accepted procedure is laid down in standard document ISO 6579:2002/A1:2007 with a sensitivity of 1 CFU per 25 g food analysed (ISO, 2007). The method consists of four successive stages: (i) pre-enrichment of 25 g food matrix in 225 ml non-selective buffered peptone water (BPW) for 18 h ± 2 h at 37°C ± 1°C, (ii) enrichment in two different selective liquid media, firstly RVS for 24 h ± 3 h at 41.5°C ± 1°C and secondly MKTTn after 24 h ± 3 h at 37°C ± 1°C, (iii) plating out and identification on XLD agar after 24 h ± 3 h incubation at 37°C ± 1°C and one selective agar plate medium of free choice, (iv) confirmation of identity by appropriate biochemical and serological tests. The complete procedure takes 4-6 working days. The ISO Standard 6579 also includes an annex D to detect *Salmonella* spp. in animal faeces and in samples from the primary production stage using selective enrichment agar MSRV. MSRV was originally developed as a rapid and sensitive test for isolating motile salmonellae from food products (De Smedt et al., 1986). Later, it was shown that this agar is preferentially useful for the isolation of motile salmonellae from faeces samples (De Zutter et al., 1991). It is planned to extend ISO Standard 6579 in three parts: the horizontal method, enumeration by a miniaturized most-

probable-number (MPN) technique, and a serotyping method for *Salmonella* (Kirsten Mooijman, EURL-Salmonella, personal communication). Recently, the second part, ISO/TS 6579-2, for the enumeration of salmonellae was published (ISO, 2012) (section 1.4.2).

Another similar, widely applied traditional culture method for the detection of *Salmonella* spp. is provided by the Food and Drug Administration in the Bacteriological Analytical Manual especially intended for use in the United States (Andrews and Hammack, 2011). The protocol gives detailed instructions for sample preparation of various food items. The selective enrichment is done using RV, modified RV, SC, or MKTT broth. Selective plating is performed on HE agar, XLD, XLT4, double-modified lysine iron agar, bismuth sulfite, triple sugar iron and/or brilliant green sulfa agar followed by biochemical species identification systems.

An alternative to shorten the isolation procedure of *Salmonella* is to use chromogenic agar media as substitutes for selective media and plates of the standard methods. In a comparative study of three chromogenic plating media (i.e. AES *Salmonella* Agar Plate, Oxoid *Salmonella* Chromogen media, and Miller-Mallinson agar, with brilliant green) with XLD- and XLT4-agar for their ability to isolate *Salmonella* colonies showed that Miller-Mallinson agars performed better than those of all the other selective media tested (Schönenbrücher et al., 2008). The comparison of the chromogenic media with ISO Standard 6579:2002 showed similar performance when large numbers of presumably naturally contaminated samples were tested (Schönenbrücher et al., 2008).

1.4.2 Enumeration of *Salmonella* with culture methods

Usually, the generation of quantitative data is laborious and time consuming. Currently, enumeration of bacteria is based on traditional bacteriological methods such as the MPN test or cell count using agar plates. Faster, easier and cheaper enumeration methods could provide risk assessors with quantitative data for improving the assessment studies. Nevertheless, knowing the quantitative *Salmonella* level during the production process could be an important hint at the source of contamination.

For enumeration of salmonellae, diagnostic laboratories need a highly sensitive and specific method because usually the number of cells in matrices from the primary production or ready-to-eat food is low and background flora may strongly influence the growth of the target bacteria. Currently, the conventional MPN test is particularly useful

for the determination of low concentrations of microorganisms and provides confirmed results within 4 to 5 days (Blodgett, 2010). Here, triplicates or five-fold replicates are prepared from ten-fold serial dilutions. All samples are then tested by the horizontal culture method. A MPN/g value can be deduced from the ratio of positive to negative samples in relation to their concentrations.

The MPN method assumes that bacteria are distributed randomly within the sample and are separated (not clustered together). The growth medium and conditions of incubation have been chosen so that a single viable cell can be multiplied and detected. For *Salmonella*, ISO/TS 6579-2 (Technical Specification) based on a mini-MPN technique was recently published (ISO, 2012). It describes a miniaturized version of the standard MPN method with less manual handling and reagents (Fravolo et al., 2003). The selective medium is MSRV and the detection limit approximately 1 CFU/g but this can vary in respect to the *Salmonella* strain and matrix applied. This is a 25-fold lower sensitivity compared to the culture detection method. If higher sensitivities are required, a conventional MPN has to be performed.

In comparison, higher levels of *Salmonella* cells (10^2-10^3 CFU/g) can be determined by the classical colony-count method using for the direct isolation selective agar such as XLD. One problem with classical colony-count is that high levels of background flora can disturb the growth of the target cells and lead to colony misidentification. In addition, selective media may inhibit the growth of stressed cells. Because of the low sensitivity direct isolation has been combined with concentration procedures (Humbert, 2002).

1.4.3 Conventional PCR for detection of *Salmonella*

Undoubtedly PCR has been established, since its invention in the mid-1980s by Kary Mullis, as the most important diagnostic technique in clinical and food microbiology. To establish PCR as a successful and reproducible tool for the detection of foodborne pathogens, assays have to meet several general requirements (Hoorfar and Cook, 2003; Malorny et al., 2003a; ISO, 2005); Most important is the consideration of an internal amplification control (IAC) which became mandatory for diagnostic assays (Hoorfar et al., 2003). An IAC is a non-target DNA sequence present in the same sample reaction tube which is co-amplified simultaneously with the target sequence. In a PCR without an IAC, a negative response (no band or signal) can indicate malfunction of the thermal cycler, PCR inhibitory substances (e.g. from sample matrix) or pipetting errors. Conversely, in a PCR

with an IAC, a positive signal will be produced when there is no target sequence but amplifiable DNA from IAC present.

The first conventional PCR assay (agarose-gel-based fragment visualisation) for specific detection of *Salmonella* DNA was published by targeting the *oriC* gene (Widjojoatmodjo et al., 1991). One year later Rahn et al. (1992) published a PCR assay based on the *invA* gene located within the highly conserved *Salmonella* pathogenicity island 1. A comprehensive set of 630 *Salmonella* and 142 non-*Salmonella* strains were tested. Only two *S. enterica* serovars Senftenberg and Lichtfield strains were negative. Nevertheless, this target and the primers showed the highest selectivity in a comparison study (Malorny et al., 2003b) and were internationally validated (Malorny et al., 2003b; Malorny et al., 2003c). The validated PCR assay includes for the first time an internal amplification control (IAC). Many other primer sets have been published differing in their target genes, detection limit and accuracy (Aabo et al., 1993; Bej et al., 1994; Kwang et al., 1996; Cohen et al., 1996; Bäumler et al., 1997; Makino et al., 1999; Ziemer and Steadham, 2003). In some cases, the strain collections used for validation did not include all seven known subspecies of *S. enterica* and *S. bongori* and did not detect epidemiologically important serovars.

1.4.4 Real-time PCR-based detection methods

In the early 1990s, the "second" generation of PCR technologies was introduced by the use of fluorescent ds-DNA dyes or DNA probes where PCR reaction and detection occur in a single-step, closed-tube procedure. This new technology is called real-time PCR, since it is possible to record the increase of PCR product on-line in a closed-tube format, reducing the risk of contamination leading to false-positive results. Data are collected throughout the PCR process, rather than at the end of the PCR, allowing accurate quantification of target sequences over at least six orders of magnitude. The principle of real-time PCR relies on fluorescence measurement during the PCR run. There are two main categories: those employing intercalating ds-DNA dyes and those using fluorescence resonance energy transfer (FRET) sequence-specific hybridization probes. The most commonly used intercalating ds-DNA dye is SYBR Green. This cyanine dye has essentially no fluorescence of its own, but when it binds to the minor groove of DNA it becomes intensively fluorescent following irradiation of light (Wittwer et al., 1997). SYBR Green binds non-specifically to ds-DNA, thereby detecting all types of ds-DNA. However, the use of a melting curve analysis after amplification can discriminate mostly between specific and unspecific PCR products. If highly specific PCR assays are necessary FRET

probe based formats must be employed. FRET is used in a number of formats to provide specific homogenous detection of PCR amplification products. The most common FRET probes used are hydrolysis probes or TaqMan probes (Livak et al., 1995) and hybridization FRET probes (Wittwer et al., 1997). Hydrolysis probes (TaqMan) use a short oligonucleotide of 20-30 bases coupled with two fluorescent dyes, a reporter dye on the 5' end and a quencher dye attached to the 3' end. The quenching dye absorbs the fluorescence from the reporter preventing the light signal from reaching the detector. During amplification, the 5' nuclease activity of the DNA polymerase hydrolyses the probe bound to the target amplification product. The released reporter dye is no longer quenched and can be detected. Hybridization FRET probes hybridize in close proximity to each other (1-5 bp spacing), so that the donor and acceptor dye provide sterical confirmation. In this conformation FRET can occur, resulting in enhanced fluorescence signal of the acceptor dye which serves as reporter dye in this system.

The first real-time PCR based assay for the detection of *Salmonella* in food was published in 1997 (Chen et al., 1997a). The assay used a specific TaqMan probe. Validation data presented by testing *Salmonella* and non-*Salmonella* pure cultures, as well as potentially naturally contaminated chicken carcass rinses, milk and pork indicated that the real-time assay is highly selective and accurate (Chen et al., 1997a; Chen et al., 1997b). Later an IAC was included and validation extended (Kimura et al., 1999). Since the primer and probe sequences were not published, the assay is not further applicable. Later, many other assays were published using the *invA* gene as target but different primers and probes were designed. A well-validated *invA* assay including an IAC using FRET hybridization probes in combination with the Light Cycler technology (Roche Diagnostics) was published by Perelle et al. (2004). Sensitivity and specificity were found to be excellent when testing fish, minced meat and raw milk.

A number of other real-time PCR assays for the detection of *Salmonella* spp. in food have been published targeting other specific fragments than *invA* (Malorny et al., 2009). Some assays were not sufficiently validated or an IAC was not developed. Those assays should be applied with care unless further validation data are available. Tartavarthy and Cannons (2010) developed a real-time PCR on the porin encoding *ompF* gene using a TaqMan probe. An extensive selectivity test was performed on pure cultures with 100% selectivity but only six artificially contaminated samples were tested and an IAC was not included in the assay. Similarly, another assay targets the *stn* gene (encoding an enterotoxin protein) of

a wide range of different *Salmonella* serovars resulting in 100% exclusivity and 96.4% inclusivity (Moore and Feist, 2007). However, food samples were not tested and an IAC was not used. Assays with an IAC included and extensively validated were based on the *fimC* gene (Piknová et al., 2005; Krascsenicsová et al., 2008), *ssrA* (McGuinness et al., 2009), *ssaN* (Chen et al., 2010) or *hilA* gene (McCabe et al., 2010; McCabe et al., 2011; Prendergast et al., 2012). The *hilA* real-time PCR method was extensively validated including by a collaborative inter-laboratory study. Subject area 1 of this thesis focuses on the development and validation of a new real-time PCR method for the detection of *Salmonella* in food using the *ttr*-operon as target.

1.4.5 Pre-PCR-sample preparation

For rapid detection of *Salmonella* in food by molecular methods the target pathogen has to be detected in a large variety of food matrices as sensitively as the standard culture method (ISO 6579:2002) is able, usually 1 cell in 25 g of food. Although PCR is sensitive enough to detect one single copy of DNA in the reaction tube, in food analysis enrichment of the target pathogen and sample preparation prior to PCR is still needed to multiply and concentrate the cells to a level applicable in the analytical assay (Feng, 2001; Löfström et al., 2004). Although enrichment is a limitation in terms of method speed, it provides essential benefits, such as diluting the effects of inhibitors, allowing the differentiation of viable from non-viable cells and allowing the repair of cells stressed or injured during food processing. Sublethally injured *Salmonella* cells, especially in food with low water activity, might have a lag phase of up to 10 h or more in which the detection of *Salmonella* is not expected (Stephens et al., 1997). A reliable pre-enrichment procedure for *Salmonella* comprises pre-enrichment for 18 h in BPW (McGuinness et al., 2009). Nevertheless, some food matrices such as carcasses and raw meat can be analysed in a shorter period using the pre-enrichment broth only (Löfström et al., 2009) or in combination with an efficient DNA extraction method (Josefsen et al., 2007). The reason for this is that *Salmonella* cells on raw meat are most likely not sublethally injured as much as in heated or dried products. Additional selective enrichment of at least 6 hours after nonselective enrichment could be advantageous when samples with high microbial background flora or high concentrations of PCR inhibitors are determined (Rådström et al., 2004). The disadvantage is a prolonged analysis time.

After enrichment bacteria have to be separated from the food matrix, PCR inhibitors removed and microbial DNA released for use in PCR assays. Common PCR inhibitors

occurring in foods are proteinases (milk products), DNases, polysaccharides (faeces, oyster meat), fat, high concentrations of calcium ions (milk products, cheese) and physical factors such as low pH values (e.g. tomato products) or high microbial loads (Wilson, 1997). Some culture media (depending on the concentration) interfere in PCR themselves, such as RV medium (Rossen et al., 1992) or can disturb fluorescence reading in real-time PCR (Rossmanith et al., 2010). Biochemically based purification methods remove inhibitors and concentrate bacterial genomic DNA. Silica-membrane columns provide a convenient method for purification of DNA which is relatively free of inhibitors. Many commercial kits for DNA extraction are available, and the performance of theses kits can vary in many cases depending on the sample type, so that they should be selected with care (Elizaquivel and Aznar, 2008). Often it is sufficient to extract the bacterial DNA by thermal cell lysis in the presence of a chelating resin, e.g. 6% (w/v) Chelex 100 suspension. Many authors have shown the usefulness of this resin for DNA extraction (Malorny et al., 2003c; Vazquez-Novelle et al., 2005). Chelex 100 is a chelating resin that has a high affinity for polyvalent metal ions and therefore binds to many PCR inhibitory substances. Another biochemical method is the matrix lysis approach where complex food matrices are solubilized (Mayrl et al., 2009). However, one main disadvantage of this method is the extensive use of chemicals and enzymes which may not only result in lack of viability of the target bacteria, but also be PCR inhibitory. This problem has been overcome by using ionic liquids, a new class of organic salts (Mester et al., 2010).

Physical non-destructive purification methods in combination with the detection of *Salmonella* were developed based on the principle of bacterial cell density properties, such as the aqueous two-phase systems (Lantz et al., 1994), buoyant density centrifugation (Wolffs et al., 2007) and floatation (Wolffs et al., 2007; Löfström et al., 2010a). Flotation is based on a density gradient centrifugation. This can separate biological particles and microorganisms that differ in buoyant density due to their lower density than the medium, which allows the cells to float. It separates *Salmonella* from the food matrix and is able to distinguish between living and dead cells (Wolffs et al., 2005). Two or more layers with decreasing densities are applied on top of the sample. During moderate centrifugation cells or particles float to a level with the same density as their own. The recovered target bacterium can be directly used in PCR. The advantage of using flotation instead of buoyant density centrifugation is that it does not require extra washing steps and the sample can be withdrawn directly from the surface.

Immunological methods use *Salmonella* specific antibodies to bind and separate cells from the food matrix. The antibodies are coated on magnetic beads in order to separate them from the remaining debris using a magnet after the cells have specifically bound to the antibodies. The technique is referred to as immunomagnetic separation (IMS) and extensively used as a pre-PCR concentration step before PCR (Rijpens et al., 1999; Notzon et al., 2006). The disadvantage of immunological preparation methods is that they do influence the specificity depending on the antibodies used and also that complex matrices might interfere with the binding capacity (Eriksson and Aspan, 2007).

A number of substances have been reported that are added to the reaction tube and directly neutralize PCR inhibitors left in the DNA sample with the result of enhancement of the efficiency of PCR (Wilson, 1997; Hedman et al., 2010). It was shown that the addition of bovine serum albumin is most effective to overcome PCR inhibitory substances (Abu Al-Soud and Rådström, 2000; Rudi et al., 2004). Other facilitators used in PCR are Triton X-100, Tween 20 or betaine (Abu Al-Soud and Rådström, 2000). Another strategy to overcome PCR inhibition is to use an alternative polymerase to *Taq*. For example, *rTth* is more resistant against many food components than *Taq* polymerase (Abu Al-Soud and Rådström, 1998). Similarly, *Tth* polymerase is advantageous to detect *Salmonella* spp. in various animal feed and meat samples (Löfström et al., 2004; Josefsen et al., 2007). The optimization of the PCR chemistry presents a relatively straightforward approach with the advantage of avoiding a labour-intensive DNA sample preparation.

1.4.6 PCR-based enumeration methods

Quantitative real-time PCR for enumeration offers several advantages in terms of speed, detection limit, cost and high throughput and has been used to generate quantitative data on *Salmonella* in various matrices including pork and poultry meat (Wolffs et al., 2006; Guy et al., 2006; Seo et al., 2006, Wolffs et al., 2007). However, pre-PCR processing methods that do not include culture enrichment, such as flotation (Wolffs et al., 2007; Löfström et al., 2010), paramagnetic beads (Warren et al., 2007) and filtration (Wolffs et al,. 2006), have a limit of quantification of approximately 10^2-10^3 CFU/ml or gram of sample due to the loss of target material during the sample preparation and the small volumes analysed. This limit of quantification is usually still too high, since most samples in the food production chain are contaminated with less than 10^2 salmonellae per gram (Boughton et al., 2007; Fegan et al., 2004). Another new approach for enumeration of low numbers of *Salmonella* is part of the second subject area of this study (section 2.2). It combines a short

pre-enrichment, harvesting target cells in the log phase and absolute quantification by real-time PCR. The quantification by real-time PCR is not based on the end-point signal but rather on the exponential increase of the initial DNA amount with regard to the performed PCR cycles (Mackay, 2004).

1.5 Validation of rapid detection methods

Real-time PCR in combination with pre-PCR DNA preparation can be used as a rapid method to detect *Salmonella* spp. in food, fulfilling many criteria stipulated by legislative authorities and the food industry. The method is increasingly applied to identify *Salmonella* in potentially contaminated food samples followed by isolation of the pathogen by the traditional horizontal culture method, e.g. according to ISO 6579:2002 (ISO, 2007). Recently, a new real-time PCR ISO Standard 22119:2011 has been published describing the minimal requirements in real-time PCR when comparable and reproducible results within and between different laboratories are needed (ISO, 2011b). However, methods based on real-time PCR and applied in food analysis need to be approved by internationally accepted certification bodies such as the Association of Official Analytical Chemists (AOAC) in the USA (http://www.aoac.org), the European Validation and Certification Organisation (MicroVal) in Europe (http://www.microval.org) or the Nordic Committee on Food Analysis (NordVal) in the Scandinavian countries (http://www.nmkl.org). Certification is rather intended for commercial products to obtain official approval of new microbiological test kits whereas open-formulated detection methods are validated and accepted by scientific experts working in the field. For validation of an alternative method in food microbiology the ISO Standard 16140:2003 has been developed, which describes the minimal requirements, procedure and data analysis for comparing an alternative method with a reference method (ISO, 2003). Currently, a revised standard is being elaborated comprising several parts covering different aspects of method validation (Lombard and Leclercq, 2010; Qvist, 2011). Validating alternative detection assays comprises two steps: a comparison study of the alternative method against the reference method carried out in one expert laboratory (in-house validation), and an interlaboratory study of the alternative method against the reference method carried out in different laboratories. The in-house validation consists of determination of the detection limit, selectivity tests (inclusivity/exclusivity) on pure strains, determination of artificially contaminated samples at different concentrations and potentially naturally contaminated samples of various food categories (Hoorfar and Cook, 2003; Qvist, 2011). The

interlaboratory study has to result in data without outliers from ten participating laboratories analysing eight blind replicates each at three contamination levels (ISO, 2003).

1.6 Genotyping methods for tracing *Salmonella*

The ability to distinguish strains or phylogenetic lineages of a bacterial pathogen is a prerequisite for addressing many questions in food microbiology and epidemiology. Currently, the traditional *Salmonella* serotyping scheme according to White-Kauffmann-Le Minor is accepted worldwide as a "gold standard" for the classification of salmonellae below the subspecies level and is widely used in surveillance of the pathogen. However, genotyping methods have been successfully established within the past two decades to characterize a subset of defined strains. They can provide better discriminatory power to differentiate closely related *Salmonella* strains and give more information in respect to the genetic relatedness within the population (Wattiau et al., 2011). The choice of the method depends on the epidemiological question that needs to be answered. To study the phylogeny or global epidemiology of *Salmonella* a set of distantly related isolates must be selected and approaches that reflect the variation in those strains that accumulate relatively slowly need to be applied. Sequence variation within housekeeping genes is ideal for such studies because they are considered to be neutral in evolution and generally their function is well understood. In the past, multilocus enzyme electrophoresis assessed the allelic variation of the genes in a strain by determination of electromorphs (allozymes) of an enzyme (Selander et al., 1996). Distinctive allele profiles (multilocus enzyme genotypes) were designated as electrophoretic types. Nowadays, with easier and cheaper DNA sequencing techniques, the concept was revised by multilocus sequence typing (MLST). MLST generates allelic types from nucleotide sequences of housekeeping genes and not from electrophoretic mobilities of the enzymes they encode (Maiden et al., 1998). Usually nucleotide sequences of a set of seven housekeeping genes are used to determine the genetic relatedness on *Salmonella* serovar level (Achtman et al., 2012). Because MLST recognizes multilocus changes at DNA level it can detect phylogenetic lineages that are assigned to individual serovars. MLST has shown that serovars can originate from more than one common ancestor (termed as polyphyletic serovar).

Methods for outbreak studies and for tracing a contamination within the food chain, where it is important to differentiate *Salmonella* below the serovar level, should be highly discriminative. This is commonly achieved by looking for DNA restriction sites within the test strain, e.g. using pulsed-field gel electrophoresis (PFGE) or fluorescent amplified

fragment length polymorphism (fAFLP). PFGE is widely used and currently the method of choice for molecular subtyping *Salmonella* serovars. It has been proven to be a useful discriminatory method and was standardized by the PulseNet Consortium to share molecular epidemiologic information in real time (Swaminathan et al., 2006; Gerner-Smidt et al., 2006). A forthcoming new molecular high resolution approach is the multilocus variable-number tandem-repeat analysis (MLVA) which is based on the determination of repetitive tandem DNA units within various loci. Repeating units occur of approx. 1-100 base pairs in length. The presence of repeated sequences is a fundamental feature of all genomes (Kolpakov et al., 2003). By the slipped strand misparing mechanism the number of tandem repeats can change with each generation. The more differences in tandem repeat units within a set of loci analysed are detected, the more distantly related the strains are interpreted to be. MLVA is applicable currently for some epidemiologically important serovars including *S. enterica* serovars Typhimurium (Lindstedt et al., 2004), Enteritidis (Boxrud et al., 2007; Malorny et al., 2008), Infantis (Ross and Heuzenroeder, 2008), Typhi (Ramisse et al., 2004) and Newport (Davis et al., 2009). Because MLVA has been proven in outbreak studies as a valuable tracing tool, standardisation is in progress to make data valid and easily comparable between laboratories (Heck, 2009; Hopkins et al., 2011; Sintchenko et al., 2012).

Recently, a number of studies have used a whole-genome single nucleotide polymorphism-based approach to identify the source of outbreaks and to clarify the epidemiology of an outbreak. Especially the outbreak caused by virulent Shiga toxin (Stx)-producing *Escherichia coli* O104:H4 in Germany demonstrated the power of next-generation sequencing technologies in prospective whole-genome characterisation in the early stages of the outbreak (Mellmann et al., 2011; Grad et al., 2012). For *Salmonella*, tracing of outbreak strains by whole-genome mapping was reported for *S. enterica* serovars Montevideo (den Bakker et al., 2011) and Newport (Fey et al., 2012).

1.7 EU Food safety legislation

The food safety policy of the European Union is based on an integrated approach called “From Farm to the Fork” developed at the beginning of the 2000s. The EU established a framework for controlling and monitoring the detection, prevention and management of food safety risks. The Regulation (EC) No 178/2002 lays down the general principle and requirements of food law and procedures in matters of food safety with the aim of ensuring the quality of foodstuffs intended for human consumption and animal feed (Anonymous,

2002). Feed and food business operators are responsible for ensuring the traceability of products at all stages of the production, processing and distribution and have an obligation to withdraw unsafe food from the market. They have to fulfil general rules on the hygiene of food and feedstuffs laid down in EC Regulations No 852/2004 (Anonymous, 2004a), No 853/2004 (Anonymous, 2004b) and No 854/2004 (Anonymous, 2004c). In that respect, they have to comply with microbiological criteria laid down in EC Regulations No 2073/2005 (Anonymous, 2005b) and No 1086/2011 (Anonymous, 2011b). Microbiological criteria specify the number of sample units to be collected, the analytical method and the number of analytical units that should conform to the limits. Generally, two types of microbiological criteria are considered: food safety and process hygiene criteria. Usually, *Salmonella* in respect to food safety criteria has to be absent in 10 or 25 g in five samples. The analytical reference method which has to be applied is ISO Standard 6579:2002/A1:2007 (ISO, 2007). Alternative analytical methods, in particular rapid methods, can be used if they were validated against the reference method and certified by a third party in accordance with ISO Standard 16140 (ISO, 2003) or other internationally accepted similar protocols. In Germany, official test methods for food samples are laid down in the German Food, Commodity and Feed Act (LFGB), §64: Official collection of methods of analysis (Anonymous, 2011c), including alternative validated PCR methods for detection of *Salmonella* whose development and validation are described in this thesis (section 2.1). Generally, the LFGB adopted EC Regulation 178/2002 (Anonymous, 2002).

2. Aim of study and results

The overall aim of the thesis was to develop tools for the rapid detection and enumeration of *Salmonella* in food. Furthermore, subtyping of epidemiologically important serovars will yield a better understanding of the population structure, genetic relatedness and potential hazard of types transmitted to humans from poultry and pigs via food.

The thesis is divided into three subject areas:

1. development and validation of a diagnostic real-time PCR for detection of *Salmonella* in food,
2. enumeration of *Salmonella* bacteria in food by real-time PCR and
3. characterization of epidemiologically important *Salmonella enterica* subsp. *enterica* serovars isolated from livestock, food and humans.

Diagnostic real-time PCR methods for the specific detection of *Salmonella* in foods are increasingly used as a rapid and reliable tool for the control of contaminated samples along the food production chain. The aim of this subject area was the development and validation of an open-formula, non-patented, accurate and robust real-time PCR based method for the detection of *Salmonella* in foods. The method is to meet the requirements of a diagnostic PCR. Therefore an inter-laboratory validation study was performed enabling it to become recognized as a standardized method for the rapid detection of *Salmonella* in microbiologically diagnostic laboratories. Another diagnostic real-time assay was developed and validated in-house for specific detection of *S. enterica* serovar Enteritidis in whole chicken carcass rinses and consumption eggs. This serovar is frequently isolated worldwide from poultry and poultry products.

The second subject area uses a novel approach as proof-of-principle to obtain quantitative data suitable for risk assessment on low numbers of *Salmonella* in the pork production chain. A quantitative real-time PCR based procedure was developed for rapid and sensitive enumeration of low numbers of *Salmonella* in cork borer samples taken from pig carcasses at slaughterhouse. The method consists of short pre-enrichment in non-selective buffered peptone water (BPW), followed by thermal cell lysis of bacterial cells to extract DNA, and finally specific *Salmonella* detection and quantification by real-time PCR.

Molecular characterization of epidemiologically important *S. enterica* serovars was conducted and is presented in the third subject area. The aim was to identify clonal groups

within the serovars and to estimate their potential health risk for humans as well as the implication of food as vehicle for the transmission from livestock to humans. A DNA microarray was developed primarily to investigate the distribution of virulence and resistance determinants in *Salmonella* strains. Emphasis was placed on the *S. enterica* serovars 4,12:d:-, Paratyphi B (d-tartrate +) and Infantis, frequently isolated in Germany from poultry. *S. enterica* serovars 4,[5],12:i:-, and Derby were investigated in detail as two important serovars frequently isolated from pigs and pork. Furthermore, five predominant *Salmonella enterica* serovars Enteritidis, Typhimurium, Infantis, Virchow and Hadar isolated in Europe from humans were compared in respect to the distribution of their virulence and resistance determinants.

2.1 Development and validation of diagnostic real-time PCR for detection of *Salmonella* in food

Publication 1

Malorny, B., Paccassoni, E., Fach, P., Bunge, C., Martin, A. and Helmuth, R. 2004. Diagnostic real-time PCR for detection of *Salmonella* in food. Appl Environ Microbiol 70, 7046-7052.

Publication 2

Malorny, B., Mäde, D., Teufel, P., Berghof-Jäger, C., Huber, I., Anderson, A. and Helmuth, R. 2007. Multicenter validation study of two blockcycler- and one capillary-based real-time PCR methods for the detection of *Salmonella* in milk powder. Int J Food Microbiol 117, 211-218.

Publication 3

Malorny, B., Bunge, C. and Helmuth, R. 2007. A real-time PCR for the detection of *Salmonella* Enteritidis in poultry meat and consumption eggs. J Microbiol Methods 70, 245-251.

An open formulated diagnostic real-time PCR method was developed and validated in intra-laboratory testing against several food items (**Publication 1**). The primers and probe of the assay targets a 95 bp fragment of the *ttr* locus which is located adjacent to the *Salmonella* Pathogenicity Island 2 (SPI 2) (Hensel et al., 1999a). The locus comprises five genes organized as an operon and is highly conserved in all *S. enterica* subspecies and *S. bongori*. Genes *ttrA*, *ttrB* and *ttrC* encode the tetrathionate reductase structural proteins and the *ttrS* and *ttrR* genes encode the sensor and response regulator components of a two-

component regulatory system (Hensel et al., 1999b). It is required for tetrathionate respiration in *Salmonella*. Primer ttr-4 is located within the *ttrA* gene, whereas primer ttr-6 and *Salmonella* target (TaqMan) probe ttr-5 are located within the *ttrA* gene (Figure 1). The assay was extensively tested in respect to its detection limit, inclusivity, exclusivity, specificity, sensitivity and robustness. The inclusivity (110 *Salmonella* strains) and exclusivity (87 non-*Salmonella* strains) was 100%. So far known, *ttr Salmonella* negative strains were not yet reported. An internal amplification control was included in the assay indicating the presence of DNA polymerase inhibitors, errors in PCR components or malfunction of the thermal cycler. The detection probability was 70% when a *Salmonella* cell suspension containing 10^3 CFU/ml was used as a template in the PCR (5 CFU per reaction) and 100% when a suspension of 10^4 CFU/ml was used. A total of 110 potentially naturally contaminated food samples and artificially contaminated whole-chicken carcass rinse at four levels with *S. enterica* serovar Enteritidis and minced meat inoculated with *S. enterica* serovar Typhimurium were compared between the real-time PCR-based method and traditional culture method according to ISO 6579:2002 (ISO, 2007) resulting in 100% agreement between the two methods. No false-negative or false-positive samples were obtained by PCR.

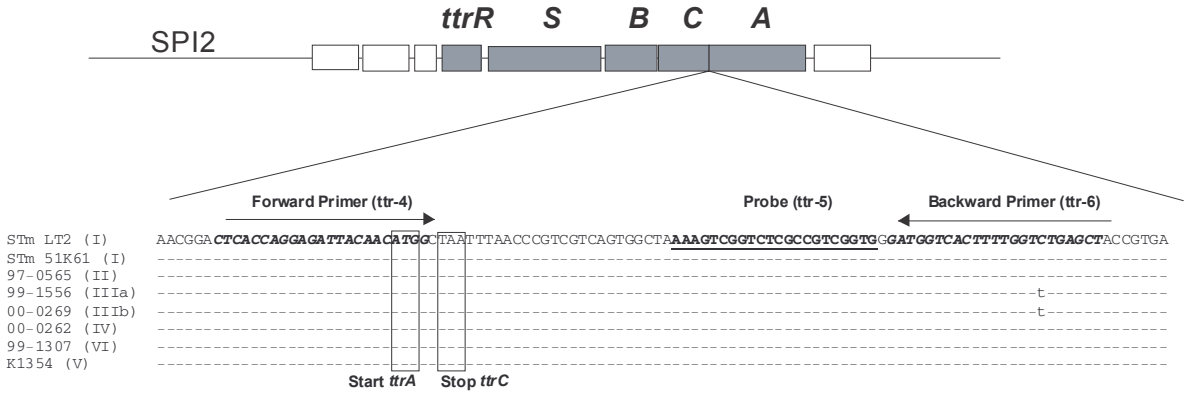

Figure 1. Schematic map of the *ttr* locus adjacent to the *Salmonella* Pathogenicity Island 2 (SPI2) and primers and probe location within the sequence region developed for real-time PCR assay for detection of *Salmonella* (**Publication 1**). A multiple alignment of the DNA region including a representative strain for each *S. enterica* subspecies is shown. A hyphen indicates an identical nucleotide with the consensus sequence. Start codon for *ttrA* and stop codon for *ttrC* are indicated by a box.

In view of the targeted acceptance of the real-time PCR (**Publication 1)** as an official test method in terms of the German Food and Feed Act (LFGB), §64 (Anonymous, 2011c), legally enabling legislative diagnostic laboratories to detect *Salmonella* by PCR in food and feeding samples, a multicenter validation study was conducted to show the robustness and accuracy of the method (**Publication 2**). Milk powder was used as an exemplary matrix. The enrichment procedure (16-20 h at 37°C in BPW) and sample DNA preparation (thermal cell lysis) prior to the real-time PCR was identical for both systems. Primers and TaqMan probe of the first assay including an IAC targets the *ttrRSBCA* gene complex (**Publication 1)**. The second assay targets the *invA* gene of *Salmonella* which is located within the *Salmonella* pathogenicity island 1. It uses previously published *Salmonella* specific primers (Rahn et al., 1992) and a newly developed TaqMan probe as well as a heterologous IAC consisting of DNA obtained from *Nicotiana tabacum*. It was thoroughly validated in-house including in many different food matrices (Anderson et al., 2011). The traditional standard culture method ISO 6579:2002 was performed in parallel in each laboratory as reference. Thirteen German food diagnostic laboratories participated in a collaborative study. Each laboratory was asked to determine 12 samples with three different artificially contaminated levels of milk powder: (i) L0 = *Salmonella* negative, (ii) L1 = < 3 MPN/g and (iii) L2 = 3,6 MPN/g. Eleven laboratories fulfilled the predefined criteria for consideration of data. Of these, 10 laboratories applied the 12 test samples on 5 different real-time PCR blockcycler models and 3 laboratories on the Light Cycler 2.0 instrument as a representative of a capillary-based real-time PCR instrument. The relative accuracy for both real-time PCR assays performed on blockcyclers was for level L0 97.5%. For level L1 the relative accuracy was 94.1% and for level L2 it was 100%. The relative accuracy on the Light Cycler 2.0 system was 100% for all levels applied to the *ttr*-real-time PCR. Despite the various enrichment conditions and real-time PCR instruments used, the standard deviation of the mean C_t values was not more than 15% for the FAM detection channel (*Salmonella* specific target) and 12% for the HEX detection channel (IAC). The mean C_t values of the target gene level L1 and L2 were 25.82 and 24.28 for the *ttr*-blockcycler PCR, 27.32 and 25.71 for the *invA*-blockcycler PCR and 30.03 and 26.15 for the *ttr*-Light Cycler 2.0 PCR (Roche Diagnostics). Altogether, the results of the collaborative trial have shown that both real-time assays are suitable to reliably detect *Salmonella* in low concentrations in milk powder.

In **Publication 3** a diagnostic real-time PCR method is described for the direct sensitive detection of *S. enterica* serovar Enteritidis and its rough variants. The serovar is implicated

in over 60% of human cases of salmonellosis in Europe and frequently isolated in poultry, especially laying hens and eggs (Gillespie et al., 2005; EFSA and ECDC, 2012). The duplex 5' nuclease (TaqMan) real-time PCR assay targets the *prot6e* gene located on the *S. enterica* serovar Enteritidis specific 60-kb virulence plasmid and the *invA* gene to indicate generally the presence of *Salmonella* DNA as a function of an IAC. A pre-enrichment step followed by a thermal cell lysis of microbial DNA prior to the assay enables the direct sensitive detection in poultry and products thereof. An in-house validation was performed on whole chicken carcass rinses and consumption eggs. The assay identified correctly 95% of the 79 *S. enterica* serovar Enteritidis strains tested comprising 19 different phage types. None of the 119 non-Enteritidis strains comprising 54 serovars was positive for the *prot6e* gene. The assay detection probability was 100% for 10^2 or more genome equivalents per reaction and 83% for 10 equivalents. Artificially contaminated whole chicken carcass rinses and eggs from hens resulted in a sensitivity of less than three CFU per 50 ml carcass rinse or 10 ml consumption egg. Non-inoculated samples were negative (> C_t 45). The comparison of 25 potentially naturally contaminated chickens showed that the accuracy compared to the traditional culture method and serotyping was 100%. Two samples were *S. enterica* serovar Enteritidis positive and three only positive for *Salmonella* spp., serotyped as *S. enterica* serovar Mbandaka. The assay is also able to reliably detect rough variants of *S. enterica* serovar Enteritidis as the second most frequently isolated serovar in laying hens in 2005 in Germany (EFSA, 2007a). These isolates have a defect in the lipopolysaccharide structure enabling serovar identification by serotyping. The application of traditional serotyping as the sole identification method in surveillance studies can therefore result in underestimation of the real prevalence of *S. enterica* serovar Enteritidis.

2.2 Enumeration of *Salmonella* bacteria in food by real-time PCR

Publication 4

Malorny, B., Löfström, C., Wagner, M., Krämer, N. and Hoorfar, J. 2008. Enumeration of *Salmonella* bacteria in food and feed samples by real-time PCR for quantitative microbial risk assessment. Appl Environ Microbiol 74, 1299-1304.

Publication 5

Krämer, N., Löfström, C., Vigre, H., Hoorfar, J., Bunge, C. and Malorny, B. 2011. A novel strategy to obtain quantitative data for modelling: combined enrichment and real-time PCR for enumeration of salmonellae from pig carcasses. Int J Food Microbiol 145 Suppl 1, S86-S95.

The enumeration of *Salmonella* bacteria at low concentrations in food is traditionally performed by the culture-based MPN test (Blodgett, 2010). With the introduction of real-time PCR an alternative method offers the possibility to enumerate bacteria indirectly by the quantification of nucleic acid of the specific pathogen. A mini-review (**Publication 4**) focuses on challenges in enumeration of *Salmonella* in food and feeding stuffs using quantitative real-time PCR and shows its potential compared to MPN. Generally, if low levels of *Salmonella* in food need to be detected, a significant time for culture enrichment is necessary to multiply bacteria to levels applicable in real-time PCR. Because cells can be damaged or stressed depending on intrinsic and extrinsic factors a prolonged time for reconvalescence can be necessary and should be taken into account. Also high levels of background flora can influence the viability of the *Salmonella* cells, especially in the presence of a small amount of the target pathogen. Another challenge is the set-up of the standard curve. The efficiency of DNA sample preparation has to be taken into consideration in a standard curve. Because the efficiency can vary depending on the food matrix, a recovery rate of the cells should be specifically determined. Furthermore, choosing DNA or cell equivalents as the unit for a standard curve must be done carefully because the interpretation might be different. Finally, in the review a concept is proposed of how *Salmonella* can be enumerated in low levels, consisting of an 8-10 h pre-enrichment step at 37°C in BPW followed by short DNA sample extraction and a quantitative real-time PCR assay based on the *ttr* target described in **Publication 1**. The novelty of the approach is to meet an adequate enrichment time where most bacteria grow in the log phase enabling quantification. It has to be long enough to achieve the required sensitivity but not so long that the growth curve reaches the plateau phase, which makes quantification impossible.

In **Publication 5** this concept is elaborated as a proof-of-principle to obtain quantitative data on low numbers of *Salmonella* in the pork chain. The method was developed and validated on artificially and naturally contaminated cork borer samples. The procedure consists of 8 h short pre-enrichment at 37°C in BPW of cork borer samples taken from chilled pig carcasses, harvesting target cells in the log phase, followed by DNA extraction and finally specific detection and quantification by real-time PCR. As specific PCR target, the *ttr* locus of *Salmonella* was selected (**Publication 1**) and a TaqMan-locked nucleic acid (LNA) probe was used, which has been reported to be more sensitive than the conventional TaqMan probe (Reynisson et al., 2006). To set up the assay, initially, a standard curve was generated by combining 118 artificially contaminated cork borer samples distributed over a

five-log range and using various *S. enterica* serovars. The regression analysis provided the regression equation y=–3.16x+27.98 (x: log inoculated CFU, y: C_t value) with R^2=0.936 and 95% CI =x±0.70. The quantification limit was 1.4 CFU/sample, the minimal number of CFU used in the inoculation experiments. The enumeration of the artificially contaminated cork borer samples using the standard curve and in parallel the MPN method as reference showed that nearly all calculated values of both methods were within the same range as the number of CFUs artificially inoculated in the sample. Both methods slightly overestimated the number of *Salmonella* cells in the samples. However, the range was compared to the respective 95% CI and determined to be acceptable.

For validation, 200 pig cork borer samples from one slaughterhouse sampled over a period of 7 weeks were qualitatively tested for *Salmonella* by real-time PCR. Twenty-eight samples were assessed to be *Salmonella*-positive and were further analysed by serotyping and quantification by both the novel enrichment PCR method and the mini-MPN technique. Altogether 26 samples were *Salmonella*-positive. The calculation of the CFU of these naturally contaminated samples using the standard curve resulted in about 10-fold lower CFU values for some of the samples. Many of the samples were negative for the MPN method while positive for the PCR method. Only three samples fell within the quantification range of the two methods. Two out of the three fell within the 95% CI and the third was close to this limit. Based on these data it is difficult to draw any conclusions. To reduce the uncertainty of the enrichment PCR method some possible changes in the pre-enrichment conditions might be considered in the future, for example prolongation of the enrichment time. The serovars frequently isolated from cork borer samples were *S. enterica* serovars Typhimurium, Derby and 4,[5],12:i:-. They were in accordance with the EU baseline survey on the prevalence of *Salmonella* in slaughter pigs (EFSA, 2008a).

2.3 Incidence and characterization of epidemiologically important *Salmonella enterica* subsp. *enterica* serovars isolated from livestock, food and humans

Publication 6

Huehn, S., Bunge, C., Junker, E., Helmuth, R. and Malorny, B. 2009. Poultry-associated *Salmonella enterica* subsp. *enterica* serovar 4,12:d:- reveals high clonality and a distinct pathogenicity gene repertoire. Appl Environ Microbiol 75, 1011-1020.

Publication 7

Huehn, S., Helmuth, R., Bunge, C., Guerra, B., Junker, E., Davies, R.H., Wattiau, P., van Pelt, W. and Malorny, B. 2009. Characterization of pathogenic and resistant genome repertoire reveals two clonal lines in *Salmonella enterica* subsp. *enterica* serovar Paratyphi B (+)-tartrate positive. Foodborne Pathog Dis 6, 431-443.

Publication 8

Huehn, S., La Ragione, R.M., Anjum, M., Saunders, M., Woodward, M.J., Bunge, C., Helmuth, R., Hauser, E., Guerra, B., Beutlich, J., Brisabois, A., Peters, T., Svensson, L., Madajczak, G., Litrup, E., Imre, A., Herrera-Leon, S., Mevius, D., Newell, D.G. and Malorny, B. 2010. Virulotyping and antimicrobial resistance typing of *Salmonella enterica* serovars relevant to human health in Europe. Foodborne Pathog Dis 7, 523-535.

Publication 9

Hauser, E., Tietze, E., Helmuth, R., Junker, E., Blank, K., Prager, R., Rabsch, W., Appel, B., Fruth, A. and Malorny, B. 2010. Pork contaminated with *Salmonella enterica* serovar 4,[5],12:i:-, an emerging health risk for humans. Appl Environ Microbiol 76, 4601-4610.

Publication 10

Hauser, E., Hebner, F., Tietze, E., Helmuth, R., Junker, E., Prager, R., Schroeter, A., Rabsch, W., Fruth, A. and Malorny, B. 2011. Diversity of *Salmonella enterica* serovar Derby isolated from pig, pork and humans in Germany. Int J Food Microbiol 151, 141-149.

Publication 11

Hauser, E., Tietze, E., Helmuth, R., Junker, E., Prager, R., Schroeter, A., Rabsch, W., Fruth, A., Toboldt, A. and Malorny, B. 2012. Clonal dissemination of *Salmonella enterica* serovar Infantis in Germany. Foodborne Pathog Dis 9, 352-360.

Of the over 2600 known *Salmonella* serovars only a few play an epidemiologically important role in humans and animals. Most of the top 10 serovars prevalent in humans are also frequently isolated in livestock, such as poultry, pigs and cattle. In contrast, some serovars are predominantly found in specific food-producing animal species but rarely cause salmonellosis in humans. **Publication 6** elucidates the contradictory situation

between the high prevalence of the monophasic *S. enterica* serovar 4,12:d:- in German broilers and the low isolation rates in humans (0.09% per year). The European baseline survey on the prevalence of *Salmonella* showed that this serovar was, with 23.6%, predominantly isolated in Germany in commercial broiler flocks of *Gallus gallus* in 2005/2006 (EFSA, 2007b). Ongoing national monitoring programmes confirmed the establishment of the serovar in the poultry production lines (BfR, 2011). For investigation, 56 strains isolated mainly from broilers and humans but also from turkeys, pigs and feed were characterized by PFGE and by a newly developed and validated DNA microarray to determine the clonality, the pathogenic gene repertoire and resistance determinants. The microarray comprises 281 oligonucleotide probes. The probes were assigned to seven different marker groups depending on the functionality of the corresponding gene sequence (number of probes): pathogenicity (83), resistance (49), serotyping (33), fimbriae (21), DNA mobility (57), metabolism (21) and prophages (13). The analysis of *S. enterica* serovar 4,12:d:- strains isolated 10 years ago and contemporary isolates showed low genetic diversity, being a sign of the persistence of a highly clonal line in German broilers. The virulence gene repertoire of *S. enterica* serovar 4,12:d:- showed that the most striking result was the complete absence of any virulence determinants encoded by prophages and the absence of plasmids in the majority of the strains. It has been proven that a virulence plasmid bearing the operon *spv* can be necessary to cause severe systemic disease (Libby et al., 1997). The *lpf* fimbrial operon encoding long polar fimbriae was also absent in all strains. Long polar fimbriae mediate adhesion to murine Peyer's patches and are required for full virulence (Bäumler et al., 1996a). The absence of several genes with known contributions to pathogenicity and metabolism which are highly prevalent in humans and animals, e.g. *S. enterica* serovars Enteritidis or Typhimurium, might be a reason for the successful spread of the serovar especially in poultry and the low prevalence in pigs, cattle and humans. Altogether, epidemiological and molecular data showed that *S. enterica* serovar 4,12:d:- can pass through the food chain from feed to poultry and occasionally finally to humans causing salmonellosis.

Interestingly, all strains investigated were susceptible to antimicrobial agents on phenotypic and genotypic level which is difficult to understand because resistance determinants can easily spread by horizontal gene transfer transmitted from cohabiting microorganisms or *Salmonella* serovars which are under similar selective pressure caused by the use of antimicrobial substances in poultry production, e.g. multidrug-resistant, (+)-tartrate-fermenting (dT+) *S. enterica* serovar Paratyphi B.

The genetic characterization of *S. enterica* serovar Paratyphi B dT+ (formerly called *S. enterica* serovar Java) and its implications for humans was part of **Publication 7**. The European baseline survey on the prevalence of *Salmonella* in commercial broiler flocks of *Gallus gallus* in 2005/2006 reveals a prevalence of the serovar in *Salmonella* positive flocks of 10.8% in Germany, 12.3% in Belgium and 18.9% in the Netherlands (EFSA, 2007). The predominant type associated with poultry in Western Europe is multidrug-resistant carrying a chromosomally located Tn*7*-like class 2 integron with a *dfrA1-sat1-aadA1* gene cassette encoding resistance against trimethoprim, streptomycin and spectinomycin (Miko et al., 2003). **Publication 7** investigated a total of 36 contemporary human, animal and environmental *S. enterica* serovar Paratyphi B dT+ strains collected from the Netherlands, Belgium, Germany and the United Kingdom in respect to their multidrug-resistance profiles and resistance determinants, clonality and pathogenicity gene repertoire using the DNA microarray described in **Publication 6.** Five monophasic *S. enterica* serovar 4,5,12:b:- strains for comparison with the biphasic Paratyphi B dT+ (4,[5],12:b:1,2) strains were included in the study.

A comparison based on the presence or absence of 104 virulence determinants (83 pathogenicity and 21 fimbrial markers) discriminated the strains into two distinct groups, differing by expression of the O:5 antigen and origin of isolation. Whereas strains isolated from chickens, pigs and humans in Germany, the Netherlands and Belgium did not express the O:5 antigen, strains isolated from various sources in the UK expressed the O:5 antigen. The two groups were clearly associated with different genetic pathogenicity gene repertoires. O:5 antigen negative strains lacked several virulence genes compared to O:5 antigen positive strains. The microarray-based population clusters could be confirmed by PFGE. A comparison of PFGE XbaI-profiles subdivided the 36 *Salmonella* Paratyphi B dT+ and five *Salmonella* 4,5,12:b:- strains again into two main groups. The first group consisted of all O:5 antigen negative strains showing an identical X8-PFGE profile. The second group was more heterogeneous with respect to the XbaI-profiles.

The two groups were also associated with different antimicrobial resistance profiles and determinants. All O:5 antigen negative strains and only one O:5 antigen positive carried a class 2 integrase gene in combination with resistance genes *dfrA1*, *sat1*(Tn*7*) and *aadA1,* as previously described by Miko et al. (2003). Twelve strains additionally carried a class 1 integrase gene. In four *S. enterica* Paratyphi B dT+ (O:5 antigen negative) strains extended-spectrum ß-lactamase genes were found ($bla_{\text{CTX-M2}}$ and bla_{TEM52}). These strains

were resistant to ceftiofur and amoxicillin/clavulanic acid, respectively. In contrast O:5 antigen positive strains showed no resistance determinants nor various other antimicrobial resistance profiles. In summary, the two *S. enterica* serovar Paratyphi B dT+ groups are phenotypically distinguishable by the expression of the O:5 antigen. On the molecular level *S. enterica* serovar Paratyphi B dT+ O:5 antigen positive strains showed a diverse genetic background compared to O:5 antigen negative strains. The distribution of the pathogenicity and resistance genes indicates that each group evolved from a different lineage and the O:5 antigen negative variant is rather associated with poultry.

Publication 8 focuses on five predominant *S. enterica* serovars (Enteritidis, Typhimurium, Infantis, Virchow and Hadar) isolated in Europe from humans, animals and food products. The study investigates the diversity of their virulence and antimicrobial resistance gene repertoire (virulotyping). Because of the high prevalence in humans the five serovars are targets in the control measure programmes in *Gallus gallus* breeding flocks in accordance with Regulation (EC) No 1003/2005 (Anonymous, 2005a). In the study a strain set of 523 *S. enterica* strains comprising the five serovars was collected from various host sources by public health and veterinary institutes across nine European countries. The strains were initially screened for the presence of ten virulence genes by PCR. Five targets (*avrA*, *ssaQ*, *mgtC*, *siiD* and *sopB*) were located on the *Salmonella* pathogenicity islands (SPIs) 1 to 5, three targets (*gipA*, *sodC1* and *sopE1*) on prophages, one (*spvC*) on the *S. enterica* serovar Typhimurium virulence plasmid and one (*bcfC*) on a fimbrial cluster. These virulence determinants represent regions known to be either highly conserved (SPIs) or variable (prophages, plasmid). Virulo-PCR screening resulted in 14 different virulence profiles (virulotypes). Most virulotypes were restricted to only one (n = 9) or two (n = 4) serovars and did not vary with host source or geographical location. A selection of 77 strains was further characterized for their pathogenicity and resistance determinants gene repertoire using the previously developed DNA microarray described in **Publication 6**. The results confirmed the virulo-PCR typing in respect to the strain distribution across Europe. By comparison of 102 virulence determinants, strains belonging to the same serovar were grouped together in a UPGMA dendrogram indicating that each serovar has a distinct virulence gene profile that was distributed across Europe. Some minor variations were found in the virulence gene complement between strains belonging to the same serovar. These differences observed within a serovar were mainly based on the distribution of prophage-encoded virulence genes but also in fimbrial clusters and virulence plasmid associated genes. For prophages this is not surprising as the acquisition and loss of such

material in bacterial genomes have been explained as a fast mode of evolution (Brüssow et al., 2004). Prophage genomes can encode additional genes, which might play a role in bacterial virulence, as in, for example, the prophages Gifsy-1, 2 and 3, Fels-1 and 2, and SopEΦ (Ehrbar and Hardt, 2005). The inclusion and reassortment of such prophage-associated virulence genes could enable *Salmonella* to adapt to different environmental conditions and to conquer new niches that might be reflected in serovar-specific ecology.

The dissemination of resistance gene profiles of the subset of 77 European strains revealed that *S. enterica* serovar Typhimurium belonging to phage types DT104 and U302 possessed the typical penta-resistance ACSSuT, encoded by the *Salmonella* genomic island 1 (Boyd et al., 2001). Whereas tetracycline resistance is encoded by *tet*(G) in these strains, in strains from *S. enterica* serovars Virchow, Hadar and Infantis the resistance is exclusively encoded by *tet*(A), often in combination with *strA*/*B* (STR resistance) and/or bla_{TEM} (AMP resistance). Resistance was not detected in *S. enterica* serovar Enteritidis, with one exception. The exceptional strain was multidrug-resistant and possibly belongs to a highly virulent clone of *S. enterica* serovar Enteritidis linked to Africa (Rodriguez et al., 2011). However, until recently antimicrobial resistance was rarely described in this serovar. Since *S. enterica* serovar Enteritidis, like serovars Hadar and Infantis, is frequently isolated from poultry in Europe, it is to be expected that selective pressure through the treatment of poultry with antibiotics would influence the uptake of resistance genes in this genome. However, it would seem that the uptake of such resistance genes by the genome of the *S. enterica* serovar Enteritidis is hampered by as yet unknown factors similarly observed in *S. enterica* serovar 4,12:d:-. In conclusion, the data support the role of mobile elements, such as prophages, plasmids and integrons, in the evolution of *S. enterica* serovars enabling the successful widespread dissemination of identical or similar virulotypes throughout the food chain across Europe.

Publications 9 and 10 focus on the *S. enterica* serovars 4,[5],12:i:- and Derby. The serovars are not exclusively adapted to pigs but are most often associated with this source in Europe. They also play an epidemiologically important role in humans. *S. enterica* serovar 4,[5],12:i:- started to emerge in the mid-1990s in Spain and reached Germany at the beginning of 2000. Between 1999 and 2011 the proportion of *S. enterica* serovar 4,[5],12:i:- isolates among all *S. enterica* isolates received by the German NRL-Salm increased from 0.1% to 12.9% with the most notable increase in 2006 and 2007. Most of the strains were isolated from pigs (approx. 50%), followed by cattle (approx. 15%),

poultry (approx. 5%) and other isolates sporadically found in the environment, wildlife and reptiles. Likewise, the number of *S. enterica* serovar 4,[5],12:i:- strains isolated from humans sent on a voluntary basis to the NRC for Salmonellae and other Enterics (RKI, Wernigerode, Germany) steadily increased from 0.1% in 1999 to 24.9% in 2011 (Erhard Tietze, personal communication). There have also been major food-borne outbreaks reported involving this monophasic serovar in humans in Europe and many non-European countries (Agasan et al., 2002; Tavechio et al., 2004; Amavisit et al., 2005; Mossong et al., 2007; Bone et al., 2010).

Publication 9 describes the results of the characterization of the monophasic *S. enterica* serovar 4,[5],12:i:- in respect to its clonality and contemporary circulating subtypes, the implication of pork functioning as a vehicle to infect humans, and the genetic relatedness to *S. enterica* serovar Typhimurium. Both serovars differ on the serotyping level in their ability to express the second phase flagellum antigen H2:1,2. Therefore, the hypothesis may be raised whether *S. enterica* serovar 4,[5],12:i:- is a monophasic variant of the biphasic *S. enterica* serovar Typhimurium with similar hazard potential for humans. Initial characterization of strains from pigs in Spain in 1997 demonstrated that the monophasic serovar lacked the *fljB* gene encoding the structural subunit of the phase two flagellum (H2-) antigen (Echeita et al., 2001). Furthermore, a DNA microarray-based typing study indicated that the monophasic serovar had a gene repertoire highly similar to that of *S. enterica* serovar Typhimurium (Garaizar et al., 2002). In my group's own study (**Publication 9**) 148 strains of the monophasic serovar isolated from pigs, pork and humans in 2006/2007 in Germany were extensively characterized by phenotypic and genotypic methods and the pathogenicity gene repertoire was compared with that of 20 *S. enterica* serovar Typhimurium strains. A main lineage of *S. enterica* serovar 4,[5],12:i:- was identified in the strains which primarily belonged to phage type DT193 and exhibited at least the tetraresistance pattern ASSuT encoded by $bla_{\text{TEM1-like}}$, *sul2*, *strA-strB* and *tet*(B), respectively. The second independently evolved lineage was phage type DT120. It was striking that 57% of the phenotypically monophasic phage type DT120 strains were positive by PCR for the structural gene *fljB*-1,2, and the two adjacent genes *fljA* and *hin*, important for phase variation of the phase two flagellum antigen, but all monophasic phage type DT193 strains lacked these genes completely. Furthermore, the phage type DT193 and DT120 strains investigated revealed a number of other different genetic properties, e.g. different clustering by PFGE and MLVA. This indicates that in Germany monophasic phage type DT120 strains have formed an additional clonal lineage different from that of

phage type DT193 strains. The most prominent PFGE XbaI-profile was STYMXB.0131, recognized in 54 monophasic DT193 strains isolated from pigs, pork and humans. The profile was associated with an outbreak of human gastroenteritis caused by consumption of pork products in Luxembourg (Mossong et al., 2007) and the clone seems to have been expanding since the middle of 2000, also in other European countries (Hopkins et al., 2010).

Sixty-one *S. enterica* serovar 4,[5],12:i:- and 20 *S. enterica* serovar Typhimurium strains were further characterized by DNA microarray. Most of the strains shared an identical pathogenicity gene repertoire and only minor variation occurred in the presence of genes usually located on the virulence plasmid pSLT. This plasmid was absent in 57 of the monophasic strains tested. Fourteen out of the 20 strains were identical to the pattern of virulence determinants found in the monophasic strains. This clearly showed that both serovars share a young common ancestor. Apparently, an epidemiological variant of *S. enterica* serovar Typhimurium, characterized by the deletion of genes responsible for the expression and phase variation of the H2-antigen and potentially surrounding genes, emerged successfully, especially in pigs and humans.

Publication 10 focused on the characterization of phylogenetic lineages belonging to *S. enterica* serovar Derby with the aim of understanding their transmission from animal to human through pork. The pathogenicity gene repertoire was compared between the lineages and to six other serovars relevant to human health in Europe (**Publications 7 and 8**). European baseline surveys revealed that the serovar is predominantly isolated in slaughter and breeding pigs in many Member States (EFSA, 2008a; EFSA, 2009). Similarly to *S. enterica* serovar 4,[5],12:i:- it is not exclusively adapted to pigs but is most often associated with this source. In humans the NRC for Salmonellae and other Enterics (RKI, Wernigerode, Germany) identified on average 0.7% of all received isolates for serotyping as *S. enterica* serovar Derby in Germany (Erhard Tietze, personal communication). Preliminary data based on MLST (http://mlst.ucc.ie/mlst/mlst/dbs/Senterica/) indicated that the serovar originates from more than one common ancestor, being called a polyphyletic serovar. Therefore, in the study the MLST scheme previously published (Kidgell et al., 2002) has been primarily used to characterize the different phylogenetic lineages occurring in the serovar. Another newly developed ST scheme was compared which was based on polymorphisms scattered in the three virulence genes *sopA*, *sopB*, and *sopD*. PFGE, VNTR-locus STTR5 sequencing and DNA

microarray were applied for subtyping strains within the different lineages. Altogether, 82 *S. enterica* serovar Derby strains were investigated that were isolated between 2006 and 2008 from pigs, pork and humans in Germany. Four major clonal groups were identified by MLST and PFGE comprising five different STs and 30 different XbaI-profiles. The most prominent group (61% of all strains) was represented by strains with sequence types ST39 (48 strains) and ST774 (2 strains). The second most prevalent group was exclusively associated with ST40 (25 strains). Strains of both groups originated from pigs, pork and humans and were approximately equally distributed among the sources. The rather distantly related groups 3 and 4 were mainly linked to human strains and none of these originated from pork. Therefore, it is unclear for these groups wether pork can function as a vehicle in their transmission to humans. By *sop* gene sequencing 12 different combinations of *sopA*, *sopB*, and *sopD* were found, which resulted in a similar tree structure representing the four clonal groups identified by MLST and PFGE. This indicates that the combination of *sopA*-*sopB*-*sopD* sequencing has potential to determine the population structure of a serovar. Six different pathogenicity array types (PATs) were detected in 32 out of the 82 *S. enterica* serovar Derby strains tested. PATs differed in up to 19 of the 102 virulence determinants investigated. The predominant types PAT DE1 and PAT DE2 differ only in the presence of the *stcC* fimbrial marker gene. Both types were exclusively associated with strains belonging to clonal group 1 (ST39) and group 2 (ST40), respectively. Tandem repeat numbers identified by VNTR sequence typing of locus STTR5 seem to be rather randomly distributed in strains and therefore not appropriate to describe the population structure of the serovar. A comparison of the pathogenicity gene repertoire of *S. enterica* serovar Derby with other epidemiologically important serovars grouped the predominant types PAT DE1 and DE2 nearest to those of *S. enterica* serovar Paratyphi B dT+ and most distantly related to those of *S. enterica* serovar Typhimurium and Enteritidis (Figure 2). This was surprising since *S. enterica* serovar Paratyphi B dT+ (O:5 antigen negative) was previously reported as a serovar associated with poultry with low pathogenicity potential for humans compared to *S. enterica* serovar Typhimurium (**Publication 7**). However, ascribing a low pathogenicity potential to *S. enterica* serovar Derby would be inappropriate since other types (PAT DE4 and PAT DE6) have more consensus in their pathogenicity gene repertoire with *S. enterica* serovars Infantis and Virchow. Nevertheless, it might be possible that some genes or genome islands important for *S. enterica* serovar Derby virulence might not be detected by the microarray. In conclusion, in Germany currently one major lineage of *S. enterica* serovar Derby is

frequently isolated from pigs and humans. As one vehicle pork has been identified and therefore poses a risk for human health.

S. enterica serovar Infantis plays a major epidemiological role in humans and animals and has been isolated in many geographically diverse regions worldwide (Hendriksen et al., 2011). In Europe, it has been the third most common serovar in humans since 2006 with a relative proportion of 1.8% in 2010 (EFSA and ECDC, 2012). It is therefore considered in the control measure programme of the EU for breeding flocks of *Gallus gallus* in accordance with Regulation (EC) No 1003/2005 (Anonymous, 2005a). Broilers and pigs were identified as one important source of infection for humans (EFSA and ECDC, 2012; Noda et al., 2010; Rajic et al., 2005). This serovar was by far the most frequently reported one in broiler meat, with 58.9% in the EU in 2010 (EFSA and ECDC, 2012). **Publication 11** investigated phenotypically and genotypically 93 epidemiologically unrelated *S. enterica* serovar Infantis strains isolated from pigs/pork, broilers/broiler meat and humans in Germany between 2005 and 2008 to gain a better understanding of the clonal structure among German *S. enterica* serovar Infantis strains currently circulating in livestock and of wether subtypes can be transmitted by pork and broiler meat to humans. PFGE revealed 35 different XbaI-profiles divided in seven different clusters. PFGE band pattern variation among the XbaI-profiles is rather low, with the lowest coefficient of similarity (*F*) of 0.72 between strains. The two most prominent clusters comprised 53 strains (cluster A) and 31 strains (cluster B) and were associated with sources from primary production of pigs and broilers, pork or broiler meat, and humans. Eleven strains representing the seven clusters shared the same sequence type ST32 by MLST. The PAT based on the presence/absence of 102 virulence determinants (**Publication 6**) was identical in 37 strains tested representing the diversity in resistance- phage-, and PFGE profiles. Twenty-three of the 37 strains were susceptible to 17 antimicrobials tested while the other were almost multidrug-resistant and mainly isolated from broilers encoding mostly an integron 1 cluster. A special resistance pattern was frequently associated with exhibiting resistance to nalidixic acid, streptomycin (encoded by *aadA1*), sulphonamide (encoded by *sul1*) and tetracycline (encoded by *tet*(A)). Compared to other *S. enterica* serovars characterized and described in **Publications 6 to 10** the genetic variation observed in *S. enterica* serovar Infantis is rather low. This indicates that *S. enterica* serovar Infantis can be regarded as a highly clonal serovar. Based on PFGE analysis two closely related genotypes were identified that are circulating in Germany in pigs, broilers, meat thereof, and humans. Consequently, these genotypes can be transmitted to humans through meat. Of public health concern is the

increasing isolation of multidrug-resistant *S. enterica* serovar Infantis in broilers. The resistance phenotype NAL STR TET SPE SMX was recognized for the first time in 2000. Meanwhile the type has been established in German broilers with a proportion of approximately 40% each year.

3. Discussion and future trends

Within the past two decades molecular microbiology has been established in food diagnostic laboratories as an important tool to detect microorganisms specifically and rapidly and to study their behaviour in food. As a key molecular-based method PCR has become an increasingly applied method in microbial detection and characterization in the past years. Furthermore, molecular typing is more and more used to understand how pathogens transmit from animals and the environment along the food chain to humans. This thesis has focused on the development and standardization of a real-time PCR method for the sensitive detection of *Salmonella* in food, to develop a new approach to quantify salmonellae in a sample and to investigate the hazard potential of specific *Salmonella* serovars for humans.

3.1 Real-time PCR for detection and enumeration of *Salmonella* in food

In Germany specific detection methods applicable to food testing are available for diagnostic laboratories according to the Food and Feed Act (LFGB), §64: Official collection of methods of analysis. The duplex 5' nuclease real-time PCR developed and validated (**Publications 1 and 2**) was accepted in 2007 as an official method in Germany (Anonymous, 2007b). It legally enables legislative diagnostic laboratories to detect *Salmonella* by PCR in food and feeding samples as an alternative method to the traditional culture based method ISO 6579:2002 (ISO, 2007). The assay has an open formula, and primers and TaqMan probe are not patented. The analysis time comprises approximately 24 hours, consisting of a pre-enrichment step in BPW overnight, an extraction-purification step for the bacterial DNA and, finally, the real-time PCR assay for the presence of *Salmonella* DNA. Whereas the *Salmonella* target signal is recallable in the FAM channel (blue dye), the IAC signal is available in a second channel detecting green dyes (YY, VIC or HEX). The use of an IAC in diagnostic PCR was becoming mandatory (Hoorfar et al., 2003). Such an IAC indicates the presence of DNA polymerase inhibitors, errors caused by PCR components or malfunction of the thermal cycler. The IAC used in this assay is a synthetically generated sequence, which is recognized by a specific probe labelled with the dye Yakima Yellow. The initial number of IAC copies in a PCR reaction has been optimized to approx. 150. Low numbers of copies reduce the competitive amplification effect between target and IAC template. On the other hand, low IAC starting copies cause unstable fluorescence signals leading to inaccurate IAC detection even in the presence of

low target copy numbers. A stable signal in a variety of *Salmonella* negative food samples, or if less than 10^4 *Salmonella* target copies are present could be assessed between 30-32 cycle numbers. The presence of larger *Salmonella* copy numbers leads to a suboptimal amplification of the IAC template due to the competitive PCR conditions. This is acceptable since the *Salmonella* signal is positive.

One major requirement of a diagnostic PCR is its robustness for successful running in different laboratories so that it can be established in other laboratories. An indication of a robust method is a high reproducibility in various laboratories. A robust real-time PCR method should be tolerant towards a range of physical and chemical parameters. The most critical parameters are usually: quality of template DNA (physical integrity of the chromosome, absence or presence of PCR inhibitors), batch differences in purity of the reagents, pipetting errors, accuracy of temperatures reached during PCR, adequacy of time duration of each PCR step, and rates of change (‘‘ramping rates’’) between the different temperatures required during amplification. For the developed *ttr*-based real-time PCR assay it was shown that a decrease in the concentrations of the PCR reagents in the master mix has only minor influence or no influence on the efficiency of the PCR at the optimized temperature of 65°C. However, a 3°C change to higher annealing-extension temperatures inhibited the reaction totally at optimized or 20% lower reagent concentrations in the reactions. In contrast, at a 20% increase of the reagent concentrations, a signal could still be detected at 68°C. This result is probably mainly caused by two parameters, temperature and magnesium ion concentration. Both factors play a major role in stabilizing primer and probe annealing (Markoulatos et al., 2002). A malfunction of the thermal cycler resulting in 3°C higher annealing-extension temperatures would therefore lead to ambiguous results under the conditions tested here.

An inter-laboratory comparison study has shown the validity of the method (**Publication 2**). The design of the study was similar to that outlined by ISO Standard 16140:2003 (also known as the MicroVal protocol) describing the minimal requirements, procedure and data analysis of comparing an alternative method with a reference method (ISO, 2003). Investigating the high reproducibility of the method by application of this protocol had led to international attention, with the consequence that the assay has been widely used and further validated in diverse other studies (de Boer et al., 2010). A modification of the assay using a LNA probe instead of a TaqMan probe was later developed (Reynisson et al., 2006; Josefsen et al., 2007) and validated on poultry faeces, carcass swabs and meat

(Löfström et al., 2009; Löfström et al., 2010b). This modified assay was approved by the Nordic Organization for Validation of Alternative Methods (NordVal) for qualitative analysis of salmonellae in raw meat samples, carcass swabs and poultry faeces (Anonymous, 2007c)

The *ttr*-based real-time PCR assay is able to detect specifically *Salmonella* DNA comprising all serovars. The detection of specific serovars is useful in terms of intervention measurements when the serovar is present in a sample. Recently, the Commission Regulation EU No. 1086/2011 (Anonymous, 2011b) has extended the criterion "*Salmonella* absence in 25 g" for fresh poultry meat for the *S. enterica* serovars Typhimurium and Enteritidis in regard to the Union target setting for the reduction of these two serovars remaining positive in 1% or less in turkey flocks and flock of broilers. For the direct detection of the *S. enterica* serovar Enteritidis in whole chicken carcass rinses and consumption eggs another real-time PCR was developed and validated in-house (**Publication 3**). The method enables the user a rapid identification of the serovar without isolation by traditional culture methods and is applicable in routine diagnostic laboratories. Food managers are then faster to assess management measurements. Furthermore, monitoring by traditional serotyping methods can result in a substantial underestimation of the real prevalence of *S. enterica* serovar Enteritidis in poultry and poultry products due to many rough LPS isolates as observed in the European baseline study on the prevalence of *Salmonella* in egg-laying flocks (EFSA, 2007a).

The real-time PCR described here showed high selectivity and accuracy. None of the 119 strains belonging to 54 different serovars tested were positive for the specific *S. enterica* serovar Enteritidis *prot6e* target sequence. However, four of the 79 *S. enterica* serovar Enteritidis strains tested were negative for the *prot6e* gene. A plasmid analysis revealed absence of the typical 60-kb virulence plasmid in theses strains. It was shown that the virulence plasmid is important in the pathogenicity of *S. enterica* serovar Enteritidis (Bakshi et al., 2003). Plasmid-free strains might be therefore less virulent. A comprehensive investigation of the frequency of the virulence plasmid in *S. enterica* serovar Enteritidis derived from animal or human isolates of different origins indicates that strains which had caused generalized infections carried the plasmid with a frequency of 98% but strains from excretors with only 57%. Poultry strains again showed 97% plasmid carriage of the cases indicating that they belong to a clone of strains which might be able to colonize deeper tissues (Helmuth and Schroeter, 1994).

The enumeration of *Salmonella* bacteria in food is usually not required in legislative regulations. Therefore the interest in standardized enumeration methods was formerly low. However, since the advent of quantitative microbial risk assessment one decade ago, there is a need for quantitative microbiological methods. Furthermore, food producers are interested in being able to measure the contamination rate of pathogens at critical control points in terms of a quality assurance program. Recently, a miniaturized MPN method has been standardized for the enumeration of salmonellae at low concentrations (ISO, 2012). Because culture-based enumeration methods are rather laborious, time-consuming and costly, alternative techniques have been developed with the introduction of quantitative real-time PCR.

As an alternative to MPN and as a proof-of-principle a novel strategy for enumeration of low numbers of salmonellae based on real-time PCR was developed; this consists of an 8 h pre-enrichment step, where most bacteria growth is in the log phase (**Publication 4**). Short DNA extraction and the analytical quantitative real-time PCR assay are then followed. The agreement in results obtained with the two methods for artificially contaminated samples was found to be satisfactory. Although the MPN method gave a higher mean value than the enrichment PCR method it was concluded that the variation is small enough to be confident that the enrichment PCR can be used in place of the reference mini-MPN method. This judgement is based on the fact that the difference in results between the methods (1.54 log CFU/sample) is in the same order of magnitude as the variation obtained for replicate analysis of the same sample using either of the two methods.

While results obtained by the real-time PCR and the MPN method showed acceptable agreement with respect to quantification in artificially contaminated samples, the calculation of CFU in naturally contaminated samples by the standard curve resulted in about 10-fold lower CFU values for some of the samples and many of the samples were negative for the MPN method while positive for the PCR method (below the LOQ). However, only a limited number of samples were included in the study (26 out of 200 samples were positive for *Salmonella*, and out of these 26 only 3 fell within the quantification range of the two methods) and therefore it is difficult to draw any conclusions based on these data.

Quantitative real-time PCR based methods have major advantages compared to the traditional MPN method for the enumeration of bacteria. PCR can generate much more data in a shorter time, thereby giving a higher degree of data confidence. The personnel

workload is tremendously lower and consequently the cost of analysis less than the counting of cells by MPN methods. Modern methodologies are faster established in laboratories, if standard documents, such that from ISO, are available. Therefore the next step is to initiate a standardization process on quantification by real-time PCR. The following recommendations should be considered in a standardization process (**Publication 5**): (i) Enrichment is necessary if salmonellae at low level are to be enumerated (approx. <500 cells per g or ml). The time of enrichment has to be adjusted to the expected status of the cells in the food/environmental matrix investigated (approximately 8 hours for meat samples). For enrichment, a non-selective broth should be used (e.g. buffered peptone water), (ii) The standard curve setup should consider the loss of nucleic acid due to the sample preparation, i.e. samples for the standard curve should be processed in the same way as the test samples. The curve should consist of at least four ten-fold serially diluted data points in duplicate. An additional data point should be included if low contaminated samples are investigated. (iii) DNA polymerase enzymes and buffers should be considered that are robust in respect to the matrix analysed and show precision in terms of the detection. It has been shown that *Tth* polymerase seems to be more appropriate for quantitative PCR than *Taq* polymerase because of its robustness and accuracy. (iv) An IAC should be included in the analytical real-time PCR assay. (v) The linear range of the standard curve should not be outside of the linear range of amplification. As an alternative approach, the non-linear part of the standard curve can be modelled.

3.2 Hazard potential of *Salmonella* serovars and subtypes

The population structure of *Salmonella enterica* was formerly regarded as clonal with a minimum of recombination events between serovars (Selander et al., 1996). However, DNA sequencing of single genes and whole-genome sequencing revealed that recombination plays an important evolutionary role in the evolution of *Salmonella* (Didelot et al., 2011). An important finding was that the traditional serological classification of *Salmonella* according to the White-Kauffmann-Le Minor scheme is not able to reflect the genetic relatedness of the strains. Several serovars consist of unrelated phylogenetic lineages of strains that do not share uniform virulence, biological or epidemiological properties and are therefore inconsistent (Achtman et al., 2012). Otherwise different serovars can also share highly similar characteristics. In this thesis, several epidemiologically important *S. enterica* serovars have been investigated by phenotypic and

genotypic methods to gain a better understanding of the relatedness, population structure, and host adaptation and to estimate the potential health risk of these for humans as well as the implication of food as vehicle for the transmission from livestock to humans. The development and application of a DNA microarray analyzing, beside other targets, pathogenicity and resistance determinants provided basic information to estimate the hazard potential of the serovars for humans (**Publication 6**). Two *S. enterica* serovars investigated, 4,12:d:- and a specific variant of Paratyphi B dT+ (O:5 antigen negative), are continuously and frequently isolated from poultry but rarely from humans in Germany (**Publications 6 and 7**). PFGE and microarray data indicate that both *S. enterica* serovars possess a highly clonal structure and spread successfully in poultry, also especially in the Netherlands and Belgium (EFSA, 2007b). It is obvious that nearly all strains analyzed of the two *S. enterica* serovars lacked genes with known contributions to pathogenicity usually encoded by prophages. Furthermore, the repertoire of fimbrial operons differed from *S. enterica* serovars which are known to be frequently isolated from humans (e.g. *S. enterica* serovars Enteritidis, Typhimurium). Especially, the *lpf* fimbrial operon encoding the long polar fimbriae LPF was absent in all strains of both serovars. It was shown that long polar fimbriae of *S. enterica* serovar Typhimurium mediate adhesion to murine Peyer's patches and are required for full virulence (Bäumler et al., 1996b). Fimbriae are generally responsible for the initial adhesion of the bacterium to the eukaryotic cells. They are frequently highly host specific and therefore an obvious factor that potentially influences host range. A number of different fimbrial clusters are encoded in a single *Salmonella* genome. The absence of several fimbrial clusters in *S. enterica* serovars 4,12:d:- and Paratyphi B dT+ (O:5 antigen negative) as well as other virulence genes is an indication for host adaptation, possibly with loss of full virulence for other hosts such as pig, cattle and humans. Apparently, those serovars possess other genetic factors which facilitate the colonization of poultry. However, host adaptation to poultry was not exclusively observed. Occasionally, the serovars were also isolated from pig, cattle or human. Host-adapted variants typically cause systemic disease in a limited number of related species, as thought for the d-tartrate non-fermenting variant of *S. enterica* serovar Paratyphi B. In contrast, host-associated or restricted variants are primarily associated with one or two closely related host species and are able to persist in the population but may also infrequently cause illness in other hosts (Wallis and Barrow, 2005). Host adaptation can be triggered by the specific organization of the immune system in birds, mammals or

cold-blooded vertebrates leading to an adapted pathogenicity gene repertoire of the serovar or variant (Bäumler et al., 1998; Kingsley and Bäumler, 2000).

Interestingly, Paratyphi B dT+ strains isolated in England from various sources and functioning as a control group revealed that these strains were distantly related to the poultry-associated Paratyphi B dT+ (O:5 antigen negative) strains and were, in respect to their PFGE profiles and pathogenicity gene repertoire, diverse (**Publication 7**). This indicates that the serovar consists of different phylogenetic groups that evolved in different niches. Recently appearing publications supported the hypothesis. It was found that the population structure of *S. enterica* serovar Paratyphi B consists of groups with divergent evolutionary paths of which one is strongly represented by poultry strains (Sangal et al., 2010; Toboldt et al., 2011). Another lineage is associated with strains isolated from reptiles and humans and yet another only with human strains (Toboldt et al., 2011). By recombination events in recent time these lineages have acquired structural surface antigens leading to their phenotypic serovar and consequently to disruption of the phylogeny within the serovar.

A striking difference between both poultry-adapted *S. enterica* serovars 4,12:d:- and Paratyphi B dT+ (O:5 antigen negative) is the encoding of antimicrobial resistance determinants in their genome. Typically, *S. enterica* serovar Paratyphi B dT+ (O:5 antigen negative) strains isolated from poultry harboured a chromosomally located class 2 integron with *dfrA1-sat1-aadA1* resistance gene cassettes conferring resistance to trimethoprim, spectinomycin and streptomycin (Miko et al., 2003; **Publication 7**). Occasionally, they possess in addition a class 1 integron located on a 128-MDa transferable plasmid with *dfrA1-aadA1* resistance gene cassette. Additional antimicrobial resistance, often in combination, has been observed against ampicillin (encoded by $bla_{\text{tem1-like}}$), nalidixic acid, kanamycin and neomycin (encoded by *aphA1*), and ceftiofur (encoded by $bla_{\text{CTX-M2}}$ and bla_{TEM52}), a 3rd generation cephalosporin. Selective pressure associated with regular medication of broilers for therapeutic or prophylactic purposes may lead to an accumulation of new resistance determinants including antimicrobial classes of critical importance for human therapy. In contrast to multidrug-resistant *S. enterica* serovar Paratyphi B dT+ (O:5 antigen negative) *S. enterica* serovar 4,12:d:- strains were completely susceptible to antimicrobial agents. Susceptibility to antimicrobial substances was also found in most *S. enterica* serovar Enteritidis strains collated from nine European countries (**Publication 8**). This is difficult to understand because resistance determinants

can easily spread by horizontal gene transfer (Schwarz and Chaslus-Dancla, 2001). Consequently, cohabitating serovars from poultry which are under similar selective pressure caused by the use of antibiotics for poultry production could disseminate resistance determinants leading to similar, if not identical, phenotypes. This observation has to be elucidated in the future. Possibly, either a genetic barrier hampers the acquisition of resistance determinants into the *S. enterica* serovar 4,12:d:- genome or the flocks infected with this serovar are not under selective pressure because of the prudent application of guidelines with low use of antimicrobial substances.

Altogether, epidemiological and molecular data show that both serovars can pass through the food chain especially from poultry to humans, occasionally causing salmonellosis. The pathogenicity gene repertoire does not currently give reasons to expect that they will pose a similar risk to consumers as other serovars frequently isolated from poultry, such as the epidemiologically most important *S. enterica* serovar Enteritidis.

S. enterica serovar Infantis is another pathogen frequently isolated in livestock, especially poultry. However, in contrast to *S. enterica* serovars 4,12:d:- and Paratyphi B dT+ (O:5 antigen negative) it also plays a major epidemiological role in humans worldwide (Hendriksen et al., 2011). In the European Union (EU) it has been the third most common serovar in humans since 2006 with a relative proportion steadily increasing from 1.0% to 1.8% in 2010 (EFSA and ECDC, 2012). Contaminated broiler meat has been identified as one important source of infection of *S. enterica* serovar Infantis for humans (Noda et al., 2010; Murakami et al., 1999) but the serovar has been also isolated frequently from pigs (Rajic et al., 2005) and cattle (Lindqvist and Pelkonen, 2007). In 2008 a baseline study in the EU revealed that *S. enterica* serovar Infantis was the most frequently reported serovar isolated from broiler carcasses, being found in 29.2% of the *Salmonella*-contaminated carcasses. Furthermore, it was at 58.9% by far the most frequently reported serovar in broiler meat in 2010 (EFSA and ECDC, 2012). An estimation for EU Member States attributed to *S. enterica* serovar Infantis responsibility for 23% of all broiler associated human *Salmonella* infections (EFSA, 2011). In pigs, the proportion of positive breeding holdings in the EU was 7.7% and of positive pig production holdings 6.1% for *S. enterica* serovar Infantis (EFSA and ECDC, 2011). In 2009 the proportion of the serovar in pork was 4.8%. The data indicate that the serovar is not adapted to specific hosts.

XbaI-PFGE analysis of 93 epidemiologically unrelated *S. enterica* serovar Infantis strains isolated in Germany between 2005 and 2008 strongly show that *S. enterica* serovar Infantis

spread in Germany from food-producing animals via food to humans (**Publication 11**). Strains isolated from the primary production of pigs and broiler chickens, broiler meat, pork and humans share the two most predominant PFGE XbaI-profiles (22.6% and 11.8%) among the 35 profiles found. These genotypes can therefore be presumed to be a hazard for human health.

S. enterica serovar Infantis can be regarded as a clonal serovar (**Publication 11**). One indication is that PFGE band pattern variation between XbaI-profiles in *S. enterica* serovar Infantis strains is rather low. The lowest observed coefficient similarity (F) between strains was 0.72. Generally, a coefficient of similarity of more than 0.70 from PFGE-types is indicative for a clonal relationship (Tenover et al., 1995; Goering, 2004). Other studies reported a similar high genetic relationship between *S. enterica* serovar Infantis strains sampled over a longer period (Dunowska et al., 2007; Lindqvist and Pelkonen, 2007). The clonality hypothesis is also supported by the observation that only one MLST and one pathogenicity gene array type was identified in all strains tested. The array type was also observed in *S. enterica* serovar Infantis strains isolated from poultry or human in other European countries (**Publication 8**). Similarly, *S. enterica* serovars 4,12:d:- and Paratyphi B dT+ (O:5 antigen negative) pathogenicity genes usually found in prophages were completely absent in *S. enterica* serovar Infantis but possessed another fimbrial cluster combination. Possibly, the different fimbrial cluster sets explain the ability of *S. enterica* serovar Infantis to colonize successfully not only poultry but also pigs and humans. The low genetic variability of *S. enterica* serovar Infantis strains might be ascribed to mechanisms protecting the serovar from major genetic rearrangements or horizontal genetic transfers, similarly to *S. enterica* serovars 4,12:d:- and Paratyphi B dT+ (O:5 antigen negative). However, another explanation could be that the serovar has a recent ancestor and was yet not able to accumulate major evolutionary changes.

Although 66% of the strains were susceptible to 17 antimicrobial substances tested, the remaining strains were almost all multidrug-resistant (two or more antimicrobial resistances) with different resistance profiles. Especially one multidrug-resistant variant (resistant against NAL, STR, SUL and TET) was found in broiler meat and pork that was previously also described in Hungary as an emerging clone (Nogrady et al., 2007). Received *S. enterica* Infantis isolates from broiler meat based on a routine diagnostic test at the NRL-Salmonella revealed that the phenotypic resistance type was recognized for the first time in 2000. Meanwhile this multidrug-resistant type has also been established in

German broilers with a proportion of approx. 40% each year. It remains to be elucidated wether other European countries were also affected. A recent study determining the antimicrobial resistance of *S. enterica* Infantis strains from Italy reported also this type within a minority of strains (Dionisi et al., 2011).

Pork is one main other source substantially contributing to human salmonellosis (Fedorka-Cray et al., 2000). It was estimated that 26.9% of infections were attributed to that source (Pires et al., 2011). The most common *S. enterica* serovars at EU level and in Germany in pigs and pork differ substantially from those found in poultry. At present the predominant *S. enterica* serovar is Typhimurium followed by Derby in most European countries (EFSA and ECDC, 2012). Since the mid-1990s a monophasic *S. enterica* serovar with the seroformula 4,[5],12:i:- has emerged in Europe and worldwide (Echeita et al., 1999; Switt et al., 2009). There have been major food-borne outbreaks reported (Tavechio et al., 2004; Amavisit et al., 2005; Mossong et al., 2007; Raguenaud et al., 2012). The serovar is most likely to be a variant of *S. enterica* serovar Typhimurium. An EU baseline survey on the prevalence of *Salmonella* in slaughter age pigs in 2006/2007 revealed that the monophasic serovar was isolated from pigs in nine of 25 participating Member States (EFSA, 2008a). At the EU level *S. enterica* serovar 4,[5],12:i:- was the fourth most prevalent serovar in slaughter age pigs. In Germany it was the second most prevalent serovar after *S. enterica* serovar Typhimurium (EFSA, 2008a).

A collection of *S. enterica* serovar 4,[5],12:i:- strains isolated from pigs, pork and humans in Germany during the years 2006 and 2007 was examined using phenotypic and molecular methods (**Publication 9**). A main lineage of *S. enterica* serovar 4,[5],12:i:- was identified in the strains which primarily belonged to phage type DT193 and exhibited at least a tetra-resistance pattern with resistance to ampicillin, sulfamethoxazole, streptomycin and tetracycline (ASSuT) chromosomally encoded by $bla_{\text{TEM1-like}}$, *sul2*, *strA*/*strB* and *tet*(B), respectively. These strains are also associated with a characteristic PFGE XbaI-profile STYMXB.0131, responsible for food-borne outbreaks (Mossong et al., 2007; Hopkins et al., 2010). The second independently evolved lineage was phage type DT120. It was striking that 57% of the phenotypically monophasic phage type DT120 strains were positive by PCR for *fljB*-1,2, *fljA* and *hin* but none of the DT193 strains. Furthermore, the DT193 and DT120 strains investigated revealed a number of other differing genetic properties, e.g. different clustering by PFGE and MLVA. This indicates that in Germany yet another major lineage is circulating within the population. The two

predominant lineages were also isolated in other European countries whereas phage type DT193 is currently the predominant one (Hopkins et al., 2010). It may be that an expansion of this clonal lineage has begun within Europe. DT193 and DT120 lineages differ from the one that initially emerged in Spain in 1997. The Spanish clone belongs to phage type U302 and displays plasmid-mediated resistance up to seven antimicrobial drugs (ACGSSuTTp) (Echeita et al., 2001; Guerra et al., 2001; García et al., 2011).

Identical traits were found in isolates from pigs, pork and humans. Consequently, the serovar is able to transmit via the food chain to humans. The impact of feeding stuff contributing to the infection of pigs remains to be elucidated. The role of feeding stuff as a contamination source is unclear but *S. enterica* serovar 4,[5],12:i:- strains from feeding stuff were not received at the NRL-Salm before 2007.

The relatedness of *S. enterica* serovar 4,[5],12:i:- to serovar Typhimurium has previously been discussed. Identical PFGE profiles in *S. enterica* serovars 4,[5],12:i:- and Typhimurium were observed (Zamperini et al., 2007). Another study suggested *S. enterica* serovar 4,[5],12:i:- as a possible monophasic variant of biphasic serovar Typhimurium phage type U302 based on comparison of PFGE and resistance profiles (de la Torre et al., 2003). Similar conclusions were outlined during investigations on isolates from Thailand (Amavisit et al., 2005). DNA-microarray based analysis has shown that *S. enterica* serovar 4,[5],12:i:- phage type U302 differs from serovar Typhimurium LT2 by five major deletions (Garaizar et al., 2002). Both serovars can also share the same multilocus sequence type (Achtman et al., 2012). In this study a comparison of 102 virulence determinants using a comprehensive set of strains clearly showed the close, almost identical, pathogenicity gene repertoire independently of whether they belonged to the monophasic or biphasic *S. enterica* serovar Typhimurium (**Publication 9**). All markers indicating fimbrial clusters occurring in *S. enterica* serovar Typhimurium were also positive in *S. enterica* serovar 4,[5],12:i:-. Nevertheless, there were some interesting genetic differences between phage type DT193 isolates of both serovars. Tetracycline resistance was mainly encoded by *tet*(B) in DT193 *S. enterica* serovar 4,[5],12:i:- strains, whereas it was encoded by *tet*(A) in DT193 *S. enterica* serovar Typhimurium strains. Additionally they clustered in different PFGE clades. Such differences indicate that the *S. enterica* serovar Typhimurium phage type DT193 lineage is not a direct ancestor of the monophasic phage type DT193. In contrast, *S. enterica* serovar 4,[5],12:i:- phage type DT120 strains showed more genetic congruence with the *S. enterica* serovar Typhimurium

phage type DT120 strains, suggesting that this biphasic subtype is the recent common ancestor of the monophasic variant. Because of the general close relationship of the monophasic variant to *S. enterica* serovar Typhimurium, in particular with respect to the pathogenicity gene repertoire and of its successful epidemiological dissemination it can be regarded as an emerging health risk for humans.

In Europe, baseline studies revealed that *S. enterica* serovar Derby is one of the most predominant *Salmonella* serovars isolated from slaughter pigs (EFSA, 2008a) and the serovar most frequently isolated from breeding pigs (EFSA, 2009). This serovar is not exclusively adapted to pigs but is most often associated with this source. Nevertheless, *S. enterica* serovar Derby is also continuously isolated from cases of human salmonellosis and from food, mainly pork, and is therefore supposed to spread from pig via pork to humans (Ling et al., 2001; Valdezate et al., 2005; Zhao et al., 2007). Outbreaks caused by *S. enterica* serovar Derby were sporadically reported worldwide especially from Japan, USA, Australia and Europe and were traced back to contaminated meat or remained without identification of the source (Rubbo, 1948; Sanders et al., 1963; Ebuchi et al., 2006; EFSA and ECDC, 2012).

In comparison to monophasic *S. enterica* serovar 4,[5],12:i:-, serovar Derby is known to have occurred in pigs for many decades (Beltran et al., 1988). Despite the frequent isolation of the serovar its phylogenetic lineages and the role of pork for transmission to humans were so far insufficiently investigated. **Publication 10** investigates 82 epidemiologically unrelated *S. enterica* serovar Derby strains isolated between 2006 and 2008 from pigs, pork and humans in Germany in respect to the transmission of clonal lineages of the serovar along the food chain. MLST and PFGE analyses identified in strains four different clonal groups. The vast majority of strains tested were assigned to clonal groups 1 (61%) and 2 (30%). These two groups differed only in one allele by MLST and belong to the same eBurst57 complex (Achtman et al., 2012). Strains originating from pigs, pork and humans and were approximately equally distributed among these sources. The data clearly show that these groups are prominent within the food chain in Germany and are transmitted from pig to human through contaminated pork. The other rather distantly related two groups 3 (ST71) and 4 (ST682) were mainly linked to human strains and none of these strains originated from pork. Since only few strains were assigned to groups 3 and 4 it is unclear whether their transmission to human can be caused by pork. It might be possible that the humans were infected by *S. enterica* serovar Derby from another

source such as birds. Based on multilocus enzyme electrophoresis typing, two divergent clone clusters of *S. enterica* serovar Derby were previously observed (Beltran et al., 1988). Both differed in their host distribution between birds and mammals including swine. However, the frequencies of human infections caused by strains from either division were found to be approximately equal, suggesting both mammals (swine) and birds as a reservoir for distinct but pathogenic *S. enterica* serovar Derby.

The pathogenicity gene repertoire of 35 *S. enterica* serovar Derby strains investigated was, in comparison with other serovars (**Publications 6 to 11**), the most diverse. Variations have been not only found in prophage located pathogenicity genes but in SPIs, some islets and fimbrial clusters resulting in altogether six PATs. The composition pathogenicity genes were also strongly associated with the four clonal groups identified by MLST and PFGE and might reflect different potential habitats for *S. enterica* serovar Derby as mentioned above. A comparison of the pathogenicity gene repertoire of the predominant types PAT DE1 and DE2 grouped them nearest to those of *S.* Paratyphi B dT+ (O:5 antigen negative) and most distantly related to those of *S. enterica* serovar Typhimurium (Figure 2). This was surprising since *S. enterica* serovar Paratyphi B dT+ (O:5 antigen negative) was previously reported as a serovar partly adapted to poultry with low pathogenicity potential for humans compared to *S. enterica* serovar Typhimurium (**Publication 7)**. One reason is that both serovars only occasionally harbour in their genomes prophage-associated genes with known contribution to *Salmonella* pathogenicity. However, ascribing a low pathogenicity potential to *S. enterica* serovar Derby would be inappropriate since other types (PAT DE4 and PAT DE5) have more consensus in their pathogenicity gene repertoire with *S. enterica* serovars Hadar, Infantis and Virchow. Nevertheless, it might be possible that some genes or genome islands important for *S. enterica* serovar Derby virulence are not detected by the microarray.

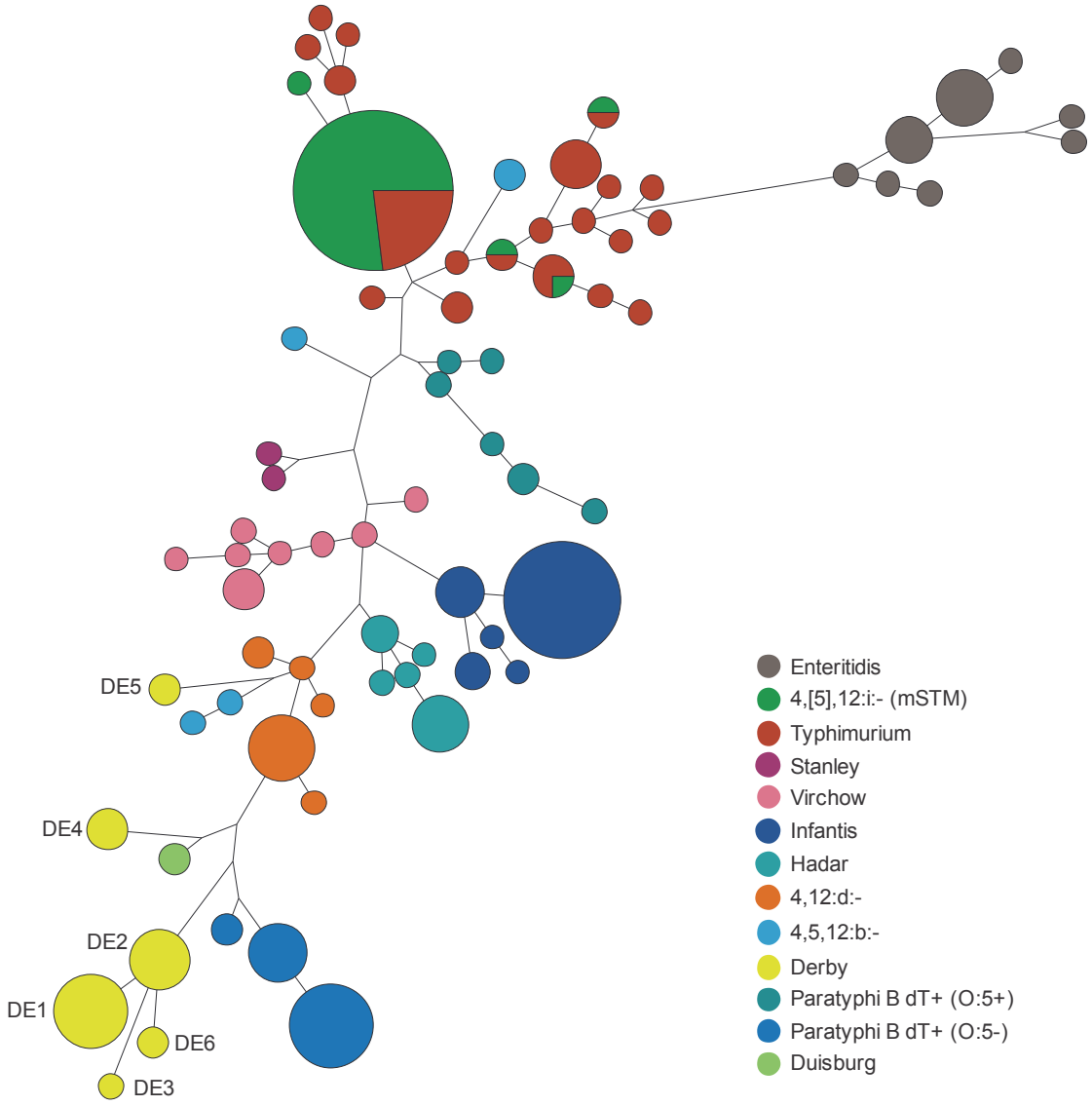

Figure 2. Maximum parsimony tree. The tree shows the differences of *S. enterica* serovars (see legend) analysed by microarray in **Publications 6 to 10** (285 strains) based on the presence/absence of 102 virulence determinants. A circle indicates a specific set of determinants (PAT). The size of the circles shows the relative number of strains related to one PAT (smallest circle one strain, largest circle 69 strains (*S. enterica* serovar Typhimurium and 4,[5],12:i:-). The length of distance lines between PATs displays the "character state changes" between them (shortest line one change, longest line 11 changes). For *S. enterica* serovar Derby (n=32) the PAT designations DE1 to DE6 are shown.

The typing of pathogenicity genes and antimicrobial genes using DNA arrays or high-through-put PCR is a molecular approach used to gain a better understanding of bacterial epidemiology and how various pathogenicity gene repertoires reflect bacterial properties such as host specificity and distribution of pathogenicity and antimicrobial resistance

determinants. In **Publication 8** the approach was applied to 523 strains belonging to five predominant *S. enterica* serovars Enteritidis, Typhimurium, Infantis, Virchow and Hadar frequently isolated in Europe from humans (EFSA and ECDC, 2012). All are known to induce gastroenteritis in a broad range of unrelated host species. By a combination of pre-PCR screening and detailed DNA microarray screening with the array developed in **Publication 6** it was striking that all strains belonging to each serovar grouped together based on the presence/absence of 102 pathogenicity genes collated on a subset of 77 strains. There were, however, some differences in the virulence gene complement between strains belonging to an individual serovar. This variation occurred primarily within those pathogenicity genes which were prophage-encoded, in fimbrial clusters or located on the virulence plasmid. For prophages this is not surprising as the acquisition and loss of such material in bacterial genomes has been explained as a fast mode of evolution (Brüssow et al., 2004).

For both *S. enterica* serovars Typhimurium and Enteritidis, 13 fimbrial clusters have been identified (McClelland et al., 2001; Thomson et al., 2008). A core set of fimbrial determinants (including *bcf*, *agf*, *csg*, *fim*, *lpf*, *saf*, *stb*, *stf*, and STM4595) is common among the five serovars investigated. Such a common set of adherence determinants would contribute to the colonization of a broad range of animal species and humans. Conversely, the absence of some fimbrial determinants is expected to contribute to host-restriction and, possibly, lower outbreak potential. For example, the *S. enterica* serovars 4,12:d:- and Paratyphi B dT+ (O:5 antigen negative) are highly associated with poultry but seldom causes illness in humans (**Publication 6 and 7**). Both *S. enterica* serovars lack the *lpfD* gene of the long polar fimbrial cluster LPF required for full virulence (Bäumler et al., 1996b) but such strains exhibit no significant defect in the colonization of chickens (Allen-Vercoe and Woodward, 1999).

For public health risk managers, it is important to know the proportion of the total incidence of a pathogen attributable to foods, and which foods are contributing to that fraction. Source attribution is a tool to answer those questions (EFSA, 2008c). The thesis clearly shows that *Salmonella* strains differentiated only on serotypic level do not reveal enough information to estimate the potential hazard for humans and the significance of livestock and food as vehicles to infect humans. In fact, molecular-driven epidemiological analysis can better reveal the association of genotypes with specific sources and their role in human *Salmonella* infections. Source attribution studies should therefore obligatorily

take into account molecular subtyping and data of resistance and pathogenicity determinants. As an example *S. enterica* Paratyphi B has been recognized by genotypic methods as a polyphyletic serovar clustering in different distantly related lineages (**Publication 7**; Toboldt et al., 2012). Several subgroups within epidemiologically important serovars have been identified that are able to transmit from livestock to humans via meat and are presumed to be a hazard for human health. Some of the subgroups are multidrug-resistant. In combination with a broad range of pathogenicity genes they are of concern for public health, especially when the prevalence rises within a short period of time in livestock and humans, as observed for the multidrug-resistant *S. enterica* serovar 4,[5],12:i:-, a monophasic variant of the biphasic *S. enterica* serovar Typhimurium (**Publication 9**). For these serovars it is essential that interventions are introduced at farm level and in the food chain in order to minimize transmission to humans. Meanwhile, in Europe, the monophasic variant is considered an emerging health hazard for humans, linked to the consumption of contaminated pork (**Publication 9**). Consequently, it has been recently included in the European *Salmonella* control measure systems (EFSA, 2010; Anonymous, 2011a).

3.3 Future trends

Currently, in most routine laboratories diagnostic procedures and surveillance of *Salmonella* are based on a wide range of traditional culture methods for isolation and subsequent phenotypic differentiation by biochemical, serological, antimicrobial resistance test and phage lysation schemes. In recent years for detection real-time PCR is now widely accepted as an alternative to the culture-based method and, for species and subspecies identification of isolates MALDI-TOF mass spectrometry is increasingly applied because of its easy handling, rapidity and cost-efficiency. Usually, only a minority of samples or isolates are further investigated by different molecular-based typing methods, e.g. in the case of outbreak studies, source attribution or other epidemiological studies. Within the past two years next-generation sequencing has begun to transform microbiology. By this technology high-throughput sequencing enables scientists to sequence genomes from any microorganism with reasonable time and cost. The outcome on information by whole-genome sequencing now already justifies by far the cost per genome sequence compared to the cost for obtaining the standard information by the individual methods such as MLST, antimicrobial resistance testing and phage typing. In a few years it is expected that rapidly falling cost and even more user-friendly high-throughput genome sequencing will lead to a

change in paradigm for microbial diagnosis epidemiology and surveillance (Köser et al., 2012). Basic routine analysis of isolates or food samples will consist of DNA genome sequencing as the first activity providing a pool of sequence data. By bioinformatic approaches the information of interest (e.g. antimicrobial resistance, virulence and SNPs) can be subsequently extracted. If necessary, phenotypic methods used on some fewer samples of interest complete the process. A challenge will be clearly the organization of how data can be shared freely worldwide in a harmonized and standardized format for investigating ongoing international outbreaks and understanding trends in population structure. Global genomic sequencing centres could help to harmonize sequencing technologies and to define standards as well as provide developing countries with samples for sequencing (Aarestrup et al., 2012). However, an outstanding investment in bioinformatic tools will be necessary to meet the requirements of diagnostic routine laboratories.

The availability of next-generation sequencing (NGS) opens furthermore a new perspective, called metagenomics, for the detection and testing of microorganisms such as *Salmonella* directly from a food sample regardless of their ability of being cultivable. This gene-based analysis method considered the whole microbial diversity within a food sample and is therefore isolate independent. The approach can contribute to gaining a deeper understanding of the interaction and biological functions of microorganisms in food and the environment which is valuable for risk assessment studies, predictive modelling and even as online testing tool for the food industry (Hoorfar et al., 2011b). However, the application of direct molecular tests without any culture-based enrichment is often not sensitive and specific enough. Therefore effective sample preparation remains a major challenge for metagenomics and also quantification of bacteria. A promising new approach, matrix lysis, is currently under development. The principle is the solubilization of the food matrix by using, detergents or organic salts (Mayrl et al., 2009; Mester et al., 2010). Volumes of 6.25 g to 12.5 g of several food matrices are reduced to small pellets appropriate for molecular DNA based methods including enumeration of bacterial cells. Last but not least molecular-based diagnosis and typing will be only applicable for diagnostic routine laboratories when they generate repeatability and reproducible data. Therefore, the validation and certification of NGS technologies remains an essential task for the future.

4. Summary

Traditional routine diagnostic microbiology on *Salmonella* consists generally of culture-based detection and identification of the microorganism on species level, and differentiation into certain phenotypes by serological methods. Further discrimination is achieved by antimicrobial resistance as well as phage typing, e.g. in the case of surveillance and epidemiology studies. In addition, within the past two decades microbial genotyping has tremendously improved our knowledge of how *Salmonella* can transmit from animals through the food chain to humans. This thesis contributes to facilitating the detection of *Salmonella* and to gaining a better knowledge of the spread of *Salmonella* from animals to humans in three respects: firstly, a rapid and sensitive detection method for *Salmonella* from food samples has been developed based on real-time PCR and extensively validated. Secondly, a new approach for low-number enumeration of *Salmonella* was developed as a proof-of-principle and thirdly, the transmission pathways of epidemiologically important *Salmonella enterica* serovars from livestock through food to humans were investigated by phenotypic and genotypic methods. By virulence and antimicrobial resistance typing in combination with epidemiological data the potential hazard for humans of certain genotypes has been estimated.

The validated real-time PCR detection method for *Salmonella*, which has been officially approved in Germany, is as accurate as traditional reference ISO 6579:2002 and places in the hands of food diagnostic laboratories a rapid, robust and cost-effective screening tool. Primers and TaqMan probe target specifically sequences within the *ttr* locus of *Salmonella*. The analytical assay was the basis for the quantification of salmonellae in low numbers in cork borer samples from pigs in combination with an eight-hour enrichment step where most bacterial growth is in the log phase. The proof-of-principle can be easily applied to other food samples by adaptation of the enrichment time.

The characterization of the pathogenicity gene repertoire of two frequently isolated *S. enterica* serovars, 4,12:d:- and multidrug-resistant Paratyphi B d-tartrate + (O:5 antigen negative), from poultry estimated the potential hazard for humans as rather low compared to that of other broad-host-range *S. enterica* serovars (e.g. Enteritidis). In contrast, *S. enterica* serovars 4,[5],12:i:- and Derby frequently found in pigs and transmitted by the one vehicle pork could be identified to be a major infection source for humans. The prevalence of *S. enterica* serovar 4,[5],12:i:- has continuously increased especially in pigs

and humans since the beginning of the 2000s. Two different subtypes of the monophasic serovar have been identified as prevalent in Germany. The genetic background of the subtypes and serovar is highly similar to that of *S. enterica* serovar Typhimurium but lacking functional genes in the genome encoding the phase two flagellum antigen H2:1,2.

Overall certain subtypes or clonal lineages of the *S. enterica* serovars investigated, some of them highly multidrug-resistant, represent a risk for human health through transmission by food. To prevent these serovars from entering the food chain and from the potential dissemination of antimicrobial resistance determinants to related microorganisms, both the farm and food production levels should be subject to rigorous *Salmonella* control measures.

5. Zusammenfassung

Die mikrobiologische Routinediagnostik für *Salmonella* besteht traditionell aus Kultur-basierten Nachweisverfahren und der Identifizierung des Mikroorganismus auf Spezies-Level, sowie der Differenzierung in bestimmte Phänotypen durch serologische Verfahren. Eine weitergehende Diskriminierung, z.B. im Fall von Überwachungs- und epidemiologischen Studien, erfolgt durch antimikrobielle Resistenz- als auch Phagentypisierung. Zusätzlich hat die mikrobielle Genotypisierung in den vergangenen zwei Jahrzehnten unser Wissen bedeutend verbessert, wie *Salmonella* vom Tier über die Lebensmittelkette zum Menschen übertragen wird. Diese Habilitationsschrift trägt in drei Aspekten dazu bei, den Nachweis von Salmonellen zu vereinfachen und das Wissen zur Verbreitung von *Salmonella* vom Tier zum Menschen zu vertiefen: Erstens, wurde eine schnelle und sensitive Nachweismethode für *Salmonella* in Lebensmittelproben basierend auf der real-time PCR entwickelt und ausgiebig validiert. Zweitens, wurde ein neues Vorgehen als Proof-of-Principle für die Zählung von niedrigen Mengen von *Salmonella* entwickelt und, drittens, wurden die Übertragungswege von epidemiologisch wichtigen *Salmonella enterica* Serovare ausgehend von Tierbeständen über Lebensmittel zum Menschen unter Anwendung von phänotypischen und genotypischen Verfahren untersucht. Durch Virulenz- und antimikrobielle Resistenztypisierung unter Zuhilfenahme von epidemiologischen Daten wurde daraus das Gefahrenpotential bestimmter Genotypen für den Menschen abgeschätzt.

Das validierte real-time PCR Nachweisverfahren für *Salmonella*, das offiziell in Deutschland bereits annerkant ist, ist so genau wie das traditionelle Referenzverfahren nach ISO 6579:2002 und gibt Lebensmittel-bearbeitende Diagnotiklabore ein schnelles, robustes und kostengünstiges Screeningwerkzeug in die Hand. Die Primer und TaqMan Sonde erkennen spezifische Sequenzen innerhalb des *ttr* Locus von *Salmonella*. Das analytische Testverfahren war auch die Grundlage für die Quantifizierung von Salmonellen in niedrigen Mengen aus Korkbohrerproben vom Schwein in Zusammensetzung mit einem acht Stunden Anreicherungsschritt, in dem das bakterielle Wachstum zum großen Teil sich in der log-Phase befindet. Der Proof-of-Principle kann durch die Anpassung der Anreicherungszeit einfach auf andere Lebensmittelproben angewendet werden.

Durch die Charakterisierung des Pathogenitätsgenrepertoires der zwei häufig aus Geflügel isolierten *S. enterica* Serovare 4,12:d:- und multiresistente Paratyphi B d-Tartrat + (O:5

Antigen negativ) konnte das Gefahrenpotential für Menschen im Vergleich zu anderen Wirtsbereich-breiten *S. enterica* Serovare (z.B. Enteritidis) als eher gering eingeschätzt werden. Im Gegensatz dazu konnten die *S. enterica* Serovare 4,[5],12:i:- und Derby, die häufig in Schweinen gefunden werden und durch das Vehikel Schweinefleisch übertragen werden, als bedeutende Infektionsquelle für den Menschen identifiziert werden. Die Verbreitung des *S. enterica* Serovars 4,[5],12:i:- ist insbesondere in Schweinen und Menschen seit Anfang der 2000er kontinuierlich angestiegen. Zwei verschiedene Untergruppen des monophasischen Serovars werden in Deutschland häufig beobachtet. Der genetische Hintergrund der Untergruppen bzw. des Serovars ist sehr ähnlich zu dem von *S. enterica* Serovar Typhimurium, es fehlen jedoch funktionelle Gene im Genom, die das Phase 2 Flagellum Antigen H2:1,2 kodieren.

Insgesamt repräsentieren bestimmte Untergruppen oder klonale Linien von den untersuchten *S. enterica* Serovaren, einige von ihnen hoch multiresistent, ein Risiko für die menschliche Gesundheit durch die Übertragung über Lebensmittel. Um den Eintritt dieser Serovare in die Lebensmittelkette und die mögliche Übertragung von antimikrobiellen Resistenzdeterminanten auf verwandte Mikroorganismen zu verhindern, sollten Erzeuger- und Lebensmittelproduktionsebenen rigorose *Salmonella* Kontrollmaßnahmen unterliegen.

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7. Abbreviations and definitions

ACSSuT	Ampicillin-chloramphenicol-streptomycin-sulphonamides-tetracycline
ACGSSuTTp	Ampicillin-chloramphenicol-gentamicin-streptomycin/spectinomycin-sulphonamides-tetracycline-trimethoprim
AOAC	Association of Official Analytical Chemists
API	Analytical profil index
BfR	Federal Institute for Risk Assessment (Bundesinstitut für Risikobewertung)
bp	Base pair
BPW	Buffered peptone water
BVL	Federal Office of Consumer Protection and Food Safety (Bundesamt für Verbraucherschutz und Lebensmittelsicherheit)
CDC	Centers for Disease Control and Prevention
CFU	Colony forming unit
CI	Confidence interval
DNA	Deoxyribonucleic acid
ds	Double stranded
DT	Definite type
dT+	d-tartrate positive
ECDC	European Centre for Disease Prevention and Control
EFSA	European Food Safety Authority
EU	European Union
EURL	European Reference Laboratory
fAFLP	Fluorescent amplified fragment length polymorphism
FAM	Phosphoramidit-derivate of 6-Carboxyfluorescein
FRET	Fluorescence resonance energy transfer
g	gram
h	hours
HE	Hektoen enteric
HEX	Hexachloro-fluorescein
IAC	Internal amplification control
IMS	Immunomagnetic separation
ISO	International Organization for Standardization

LFGB	German Food, Commodity and Feed Act (Lebensmittel-, Bedarfsgegenstände-, und Futtermittelgesetzbuch)
LNA	Locked nucleic acid
LOQ	Limit of quantification
MALDI-TOF	Matrix assisted laser desorption/ionization-Time of flight
MicroVal	European Validation and Certification Organisation
MKTTn	Müller-Kauffmann tetrathionate novobiocin
ml	millilitre
MLEE	Multilocus enzyme electrophoresis
MLST	Multilocus sequence typing
MPN	Most probable number
MS	Member State
MSRV	Modified semi-solid Rappaport-Vassiliadis
NAL	Nalidixic acid
NGS	Next-generation sequencing
NordVal	Nordic Committee on Food Analysis
NRC	National Reference Centre
NRL	National Reference Laboratory
PAT	Pathogenicity array type
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PT	Phage type
RKI	Robert Koch-Institute
RV	Rappaport-Vassiliadis
RVS	Rappaport-Vassiliadis with soya
S.	*Salmonella*
SC	Selenite cystine
SCVPH	Scientific Committee on Veterinary measures relating to Public Health
SMX	Sulfamethoxazole
SNP	Single nucleotide polymorphism
SPE	Spectinomycin
STR	Streptomycin
TET	Tetracycline
UK	United Kingdom

UPGMA	Unweighted-pair group method with arithmetic averages
VIC	VIC fluorescence dye
w/v	mass/volume
XLD	Xylose lysine desoxycholate
XLT4	Xylose lysine tergitol 4
YY	Yakima yellow

8. Contribution to publications

The degree of the contribution to the publications by each author is subdivided into the following elements:

1. Formulating the scientific idea based on theoretical assumptions to be clarified, including formulation of the hypothesis to be answered through analytical word and research plans
2. Planning of experiments and analyses; design of the experimentally methods able to answer hypothesis
3. Involvement in analytical work with respect to the concrete experimental studies and investigations
4. Presentation, interpretation and discussion of the results
5. Writing first draft manuscript and reviewed versions

Publication 1

Malorny, B., Paccassoni, E., Fach, P., Bunge, C., Martin, A. and Helmuth, R. 2004. Diagnostic real-time PCR for detection of *Salmonella* in food. Appl Environ Microbiol 70, 7046-7052.

1. Malorny, Fach
2. Malorny, Paccassoni
3. Bunge, Paccassoni, Malorny
4. Martin, Malorny
5. Malorny, Helmuth

Publication 2

Malorny, B., Mäde, D., Teufel, P., Berghof-Jäger, C., Huber, I., Anderson, A. and Helmuth, R. 2007. Multicenter validation study of two blockcycler- and one capillary-based real-time PCR methods for the detection of *Salmonella* in milk powder. Int J Food Microbiol 117, 211-218.

1. Malorny, Teufel, Berghof-Jäger, Mäde
2. Malorny, Huber, Anderson
3. Malorny, Huber, Anderson

4. Malorny, Teufel, Berghof-Jäger, Mäde, Huber, Anderson
5. Malorny, Helmuth

Publication 3

Malorny, B., Bunge, C. and Helmuth, R. 2007. A real-time PCR for the detection of *Salmonella* Enteritidis in poultry meat and consumption eggs. J Microbiol Methods 70, 245-251.

1. Malorny, Helmuth
2. Malorny
3. Bunge
4. Malorny
5. Malorny, Helmuth

Publication 4

Malorny, B., Löfström, C., Wagner, M., Krämer, N. and Hoorfar, J. 2008. Enumeration of *Salmonella* bacteria in food and feed samples by real-time PCR for quantitative microbial risk assessment. Appl Environ Microbiol 74, 1299-1304.

1. Hoorfar, Malorny, Wagner, Löfström
2. Malorny, Krämer, Hoorfar
3. not appropriate
4. Hoorfar, Malorny, Wagner, Löfström, Krämer
5. Malorny, Hoorfar

Publication 5

Krämer, N., Löfström, C., Vigre, H., Hoorfar, J., Bunge, C. and Malorny, B. 2011. A novel strategy to obtain quantitative data for modelling: combined enrichment and real-time PCR for enumeration of salmonellae from pig carcasses. Int J Food Microbiol 145 Suppl 1, S86-S95.

1. Malorny, Hoorfar
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Publication 6

Huehn, S., Bunge, C., Junker, E., Helmuth, R. and Malorny, B. 2009. Poultry-associated *Salmonella enterica* subsp. *enterica* serovar 4,12:d:- reveals high clonality and a distinct pathogenicity gene repertoire. Appl Environ Microbiol 75, 1011-1020.

1. Malorny, Huehn, Helmuth
2. Malorny, Huehn
3. Huehn, Bunge, Junker
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5. Malorny, Huehn

Publication 7

Huehn, S., Helmuth, R., Bunge, C., Guerra, B., Junker, E., Davies, R.H., Wattiau, P., van Pelt, W. and Malorny, B. 2009. Characterization of pathogenic and resistant genome repertoire reveals two clonal lines in *Salmonella enterica* subsp. *enterica* serovar Paratyphi B (+)-tartrate positive. Foodborne Pathog Dis 6, 431-443.

1. Helmuth, Davies, Wattiau, van Pelt, Malorny
2. Malorny, Huehn
3. Huehn, Guerra, Junker, Bunge
4. Huehn, Malorny, Guerra, Helmuth, Davies, Wattiau, van Pelt
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Publication 8

Huehn, S., La Ragione, R.M., Anjum, M., Saunders, M., Woodward, M.J., Bunge, C., Helmuth, R., Hauser, E., Guerra, B., Beutlich, J., Brisabois, A., Peters, T., Svensson, L., Madajczak, G., Litrup, E., Imre, A., Herrera-Leon, S., Mevius, D., Newell, D.G. and Malorny, B. 2010. Virulotyping and antimicrobial resistance typing of *Salmonella enterica* serovars relevant to human health in Europe. Foodborne Pathog Dis 7, 523-535.

1. Malorny, Huehn, La Ragione, Anjum
2. Malorny, Huehn

3. La Ragione, Anjum, Saunders, Bunge, Hauser, Beutlich, Brisabois, Peters, Svensson, Madajczak, Litrup, Imre, Herrera-Leon
4. Huehn, Malorny, Woodward, Helmuth, Hauser, Guerra, Mevius, Newell, Saunders
5. Malorny, Huehn

Publication 9

Hauser, E., Tietze, E., Helmuth, R., Junker, E., Blank, K., Prager, R., Rabsch, W., Appel, B., Fruth, A. and Malorny, B. 2010. Pork contaminated with *Salmonella enterica* serovar 4,[5],12:i:-, an emerging health risk for humans. Appl Environ Microbiol 76, 4601-4610.

1. Malorny, Hauser, Tietze
2. Malorny, Hauser, Tietze
3. Hauser, Blank, Prager, Rabsch, Fruth
4. Malorny, Hauser, Tietze, Helmuth, Appel, Prager, Rabsch, Fruth
5. Malorny, Hauser

Publication 10

Hauser, E., Hebner, F., Tietze, E., Helmuth, R., Junker, E., Prager, R., Schroeter, A., Rabsch, W., Fruth, A. and Malorny, B. 2011. Diversity of *Salmonella enterica* serovar Derby isolated from pig, pork and humans in Germany. Int J Food Microbiol 151, 141-149.

1. Malorny, Tietze, Hauser
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4. Malorny, Hauser, Tietze, Helmuth, Prager, Rabsch, Fruth
5. Hauser, Malorny

Publication 11

Hauser, E., Tietze, E., Helmuth, R., Junker, E., Prager, R., Schroeter, A., Rabsch, W., Fruth, A., Toboldt, A. and Malorny, B. 2012. Clonal dissemination of *Salmonella enterica* serovar Infantis in Germany. Foodborne Pathog Dis 9, 352-360.

1. Malorny, Tietze, Hauser
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4. Malorny, Hauser, Tietze, Helmuth, Prager, Rabsch, Fruth
5. Malorny, Hauser

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10. Annex (Publications 1-11)

Publication 1

Malorny, B., Paccassoni, E., Fach, P., Bunge, C., Martin, A. and Helmuth, R. 2004.

Diagnostic real-time PCR for detection of *Salmonella* in food.

Appl Environ Microbiol 70, 7046-7052.

http://dx.doi.org/10.1128/AEM.70.12.7046-7052.2004

Publication 2

Malorny, B., Mäde, D., Teufel, P., Berghof-Jäger, C., Huber, I., Anderson, A. and Helmuth, R. 2007.

Multicenter validation study of two blockcycler- and one capillary-based real-time PCR methods for the detection of *Salmonella* in milk powder.

Int J Food Microbiol 117, 211-218.

http://dx.doi.org/10.1016/j.ijfoodmicro.2007.04.004

Publication 3

Malorny, B., Bunge, C. and Helmuth, R. 2007.

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J Microbiol Methods 70, 245-251.

http://dx.doi.org/10.1016/j.mimet.2007.04.013

Publication 4

Malorny, B., Löfström, C., Wagner, M., Krämer, N. and Hoorfar, J. 2008.

Enumeration of *Salmonella* bacteria in food and feed samples by real-time PCR for quantitative microbial risk assessment.

Appl Environ Microbiol 74, 1299-1304.

http://dx.doi.org/10.1128/AEM.02489-07

Publication 5

Krämer, N., Löfström, C., Vigre, H., Hoorfar, J., Bunge, C. and Malorny, B. 2011.

A novel strategy to obtain quantitative data for modelling: combined enrichment and real-time PCR for enumeration of salmonellae from pig carcasses.

Int J Food Microbiol 145 Suppl 1, S86-S95.

http://dx.doi.org/10.1016/j.ijfoodmicro.2010.08.026

Publication 6

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Poultry-associated *Salmonella enterica* subsp. *enterica* serovar 4,12:d:- reveals high clonality and a distinct pathogenicity gene repertoire.

Appl Environ Microbiol 75, 1011-1020.

http://dx.doi.org/10.1128/AEM.02187-08

Publication 7

Huehn, S., Helmuth, R., Bunge, C., Guerra, B., Junker, E., Davies, R.H., Wattiau, P., van Pelt, W. and Malorny, B. 2009.

Characterization of pathogenic and resistant genome repertoire reveals two clonal lines in *Salmonella enterica* subsp. *enterica* serovar Paratyphi B (+)-tartrate positive.

Foodborne Pathog Dis 6, 431-443.

http://dx.doi.org/10.1089/fpd.2008.0221

Publication 8

Huehn, S., La Ragione, R.M., Anjum, M., Saunders, M., Woodward, M.J., Bunge, C., Helmuth, R., Hauser, E., Guerra, B., Beutlich, J., Brisabois, A., Peters, T., Svensson, L., Madajczak, G., Litrup, E., Imre, A., Herrera-Leon, S., Mevius, D., Newell, D.G. and Malorny, B. 2010.

Virulotyping and antimicrobial resistance typing of *Salmonella enterica* serovars relevant to human health in Europe.

Foodborne Pathog Dis 7, 523-535.

http://dx.doi.org/10.1089/fpd.2009.0447

Publication 9

Hauser, E., Tietze, E., Helmuth, R., Junker, E., Blank, K., Prager, R., Rabsch, W., Appel, B., Fruth, A. and Malorny, B. 2010.

Pork contaminated with *Salmonella enterica* serovar 4,[5],12:i:-, an emerging health risk for humans.

Appl Environ Microbiol 76, 4601-4610.

http://dx.doi.org/10.1128/AEM.02991-09

Publication 10

Hauser, E., Hebner, F., Tietze, E., Helmuth, R., Junker, E., Prager, R., Schroeter, A., Rabsch, W., Fruth, A. and Malorny, B. 2011.

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Int J Food Microbiol 151, 141-149.

http://dx.doi.org/10.1016/j.ijfoodmicro.2011.08.020

Publication 11

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Foodborne Pathog Dis 9, 352-360.

http://dx.doi.org/10.1089/fpd.2011.1038