Development of Live Salmonella Vaccines Optimally Attenuated for Chickens

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Introduction

Over 70% of all cases of enteritis and toxic reactions from foodstuffs are caused by salmonella (S.) (*Zastrow & Schöneberg*, 1994). In Germany, a rate of incidence of more than 200 cases of salmonella per 100,000 inhabitants, approximately 160 related fatalities and an estimated number of unknown cases (*Tagungsbericht der DGHM*, 1994; afp, 1993) led to a renewed interest in the daily press concerning the salmonella controversy.

S. enteritidis (S. e.) is at present the dominant serotype (*Albert et al.*, 1992; *Erichsen & Sullmann*, 1994; *Brown et al.*, 1994; *Schroeter et al.*, 1995). It has been frequently detected in illnesses arising from the consumption of raw egg dishes that were improperly prepared, kept too long in storage, and incorrectly stored (*Vielitz et al.*, 1994; *Schobries*, 1995).

Since the oral administration of live vacccine stimulates mucosal immunity in particular (*Hess*, 1991, *Holmgreen et al.*, 1992; *McGhee et al.*, 1992; *Walker*, 1994) orally vaccinated chickens, when subsequently infected, shed the challenge strain with lower bacterial germ counts, and in reduced period of time (*Pritchard et al.*, 1978; *Truscott*, 1981; *Suphabphant et al.*, 1983; *Coe & Wood*, 1992; *Meyer et al.*, 1993). Analogously, the same holds true for internal organs: markedly lower bacterial counts and quicker elimination of the wild type strain (*Methner et al.*, 1994). The vaccination of all exposed chicken flocks and regular hygienic measures can break the chain of infection from chicken to chicken and ultimately from chickens to humans (*Vielitz et al.*, 1992).

However, the realization of optimal vaccine action in a cost-efficient manner requires that the general principles governing the pathogen-host relationship has to be observed when developing potential vaccine strains. For the case at hand, this means that the degree of attenuation grade must be adapted to the chicken's well-known lower susceptibility to salmonella infections. Virulence should therefore not be reduced as strongly as in other vaccine strains (Table1).

Table 1: Susceptibility of various host species to S. typhimurium (S. tm.) per species and per kilogram body weight.

Mouse „mouse typhoid fever"	> calf	> chicken
LD_{50} i.p. ~ 10^1 CFU;		
~ 5 x 10^2 CFU/kg BW	-	≥ 10^{10} CFU
oral ≤ 10^4 CFU,		
≤ 5 x 10^5 CFU/kg BW	ca. 5 x 10^7 CFU/kg BW	≥10^{11} CFU

Efficacy problems concerning immunization of chicken to combat salmonella infections

The percentile distribution of the enterobacterial colonization pattern (including salmonellae with their numerous serotypes) is controlled in part by bacterial competition for the nutrient substrate and the attachment sites on the mucosal epithelia. Another decisive factor that influences bacterial colonization is the host's activated mucosal immunity.

In the process, secretory IgA inhibit attachment to the intestinal cells, thereby preventing a non-physiological and dominant colonization by a single serotype, which is usually accompanied by illness when the level of susceptibility is moderate to high (*Blatz et al.*, 1987 & 1990; *Schröder et al.*, 1990; *Blatz & Linde*, 1990; *Linde*, 1991). The consequence of this is that after a salmonella infection its colonization density within the enterobacterial flora may initially reach about values of 10%. However, after 7-10 days this drops to a magnitude of 0.1‰, a level at which flocks fail to self-eliminate this pathogen and are latent contaminated. A quantitative reduction in salmonella shedding through the stimulated mucosal immunity can be achieved by vaccination only during the colonization phase with its high bacterial counts. The resulting drop in infection stress can lead within one year to salmonella-controlled flocks (*Selbitz*, 1994). The extent of mucosal immunity and the resulting reduction in the shedding of the wild type strain is quantitatively dependent on the number of pathogenic bacteria that penetrate the intestinal lymph tissue and remain persistent for a sufficient lenght of time (*Hess*, 1992; *Holmgren et al.*, 1992; *McGhee et al.*, 1992; *Walker*, 1994).

In order to stimulate mucosal immunity resembling such one of a wild type type strain infection, salmonella vaccine strains for chickens must compensate for their lower suspectibility with a higher level of residual virulence (= lower degree of attenuation) as compared to other vaccines. As a result of a relatively high vaccine dose and simultaneously a maintained sufficient level of invasiveness (*Sansonetti*, 1993; *Amin et al.*, 1994; *Gentraux et al.*, 1994) and due to the remaining yet adequate levels of propagation and persistence *in-vivo*, these vaccines will accumulate such amounts of antigen in the intestinal wall which are comparable to such ones occuring in the course of an optimal protecting natural infection.

The term „chicken-adapted attenuation grade" is in principle a new concept of the interaction between pathogen and host. The complete loss of virulence with its accompanying absolute tolerance is deliberately foregone in favour of optimum vaccine potency, thus establishing an altered line of reference. Attenuation grade (i.e. reduced virulence) is limited to a point that still ensures invasiveness as well as a residual but adequate propagation *in-vivo* (residual virulence).

An adapted attenuation grade can be recognized in that a single parenteral dose stimulates the same immunity as compared with the oral administration of the 100-fold dosage (*Linde et al.*, 1993). The chicken-adapted attenuation grade for S. typhimurium (S. tm.), as measured in the mouse model with intraperitoneal infection, corresponds to an LD_{50} of 10^6 germs and a generation time lengthened to approximately 31-32 minutes as an attenuation equivalent (wild type strain approximately 10^1 germs, with a generation time of approximately 21-22 minutes). Any lower degree of attenuation should be avoided, for this may lead to a temporary immunosuppression similiar to that following a wild type strain infection (*Hasan & Curtis*, 1994).

The oral administration of the vaccine strains S. tm., S. e. (S. infantis and S. anatum), which were produced with this chicken-adapted attenuation grade, resulted in a reduction of wild type strain shedding by 1-2 powers of ten in the first 6-10 days following oral challenge. The salmonella colonization density subsequently levelled out at about ≤ 0.1‰ of the enterobacterial intestinal flora (*Linde et al.*, 1993; *Cooper et al.*, 1994), i.e. at levels characteristic of a latent salmonella infection.

In the past, the isolation of appropriate mutants needed for vaccine strains having any desired degree of attenuation (*Linde et al.*, 1990) has been a problem that was either unknown or unsolvable (*Doss*, 1994). This has now been solved with the aid of metabolic drift mutants.

The use of metabolic drift mutations to adapt attenuation to the susceptibility of the host species

Metabolic drift mutants are by definition pathogens having minus mutations in essential enzymes and metabolic compartments. The resulting alterations in the metabolic pathways lead to longer generation times (time required for the bacterial count to double) and to a corresponding reduction in virulence. Since they are generated by random hits, the mutations may appear at various gene locations (gene loci). Depending on the mutated gene locus involved (genotype), the resulting structural changes in the metabolic compartments may vary to a greater or lesser degree, with a corresponding greater or lesser lengthening of the generation time and corresponding reduction of virulence. Metabolic drift mutants therefore always split into a broad spectrum of clones, of which 30% to 70% - depending on the strain and selected metabolic drift - exhibit a quantitatively different high degree of attenuation (*Linde*, 1981; *Linde et al.*, 1990).

The metabolic compartments are well-known sites of action for antibiotics, from which their therapeutic use derives. The result of the mutative structural changes in the metabolic compartments is thus not only attenuation but, as a simultaneous side-effect, an antibiotic resistance brought about by the loss of antibiotic binding sites. With regard to the attenuation this resistance has no theropeutical consequences and is used only to identify the vaccine strain.

For the attenuation of the vaccine strains to be introduced, the following metabolic compartments are genetically altered by means of chromosomal antitibiotic-resistant mutations:

Metabol. compartment		Resistence to
(1) RNA polymerase	(Transcription of DNA to RNA)	Rifampicin (Rif)
(2) Gyrase	(Controls DNA replication)	Nalidixic acid (Nal)
(3) Ribosomal protein S12	(Ribosomal structure, helps control accurate polypeptide synthesis)	Streptomycin (Sm)

From the standpoint of molecular biology, a reversal of this principle means that chromosomal antibiotic-resistant mutants are always metabolic mutants as well.

At this point doubts may be arise concerning the possible transmission of the resistance *in-vivo* to other bacteria with the resulting therapeutic problems. This view is unfounded for the following two reasons:

1. With the help of gene transfer by means of transduction, it was demonstrated, that, as expected, the attenuation and resistance proceed from the same mutation and therefore is transmitted as functional unit (*Linde at al.*, in preparation). If such a gene transfer actually occur *in-vivo* — which according to direct (Kaper et al., 1994) and indirect accounts (Du Pont et al., 1971; Formal et al., 1971) are to be expected in the range of 10^{-10} — this would give rise to resistant but only avirulent bacteria.

2. It has been demonstrated that the occurrence and disappearance of these metabolic mutants always takes place spontaneously, for instance in the gastrointestinal tract. In this sense they — apart from their isolation in the laboratory and their being employed as vaccine strains — are a normal biological and evolutionary process (*Linde et al.*, in preparation).

Optimising vaccine strains using safety functions

Outer cell membrane mutation:
A cell membrane mutation serves as a third the vaccine strain optimizing marker (and at the same time, as defined by the WHO (*WHO Report*, 1972), as an attenuation marker) by increasing the cell's permeability to erythromycin and other antibiotics and noxæ (*Hancock*, 1945; *Vaara*, 1993). This mutation gives the vaccine strain three additional safety functions:

1. Shedding of the vaccine strain by chicks is limited to a maximum of 21 days (*Linde et al.*, 1993; *Hahn et al.*, 1993), see Figure 1.

2. The strain's reduced survival in the environment entails a faster extinction in the stable dung (*Linde & Randhagen*, 1986).

3. Its sensitivity to antibiotics such as doxycyclin, chloramphenicol and others used for human therapy is increased fourfold; for the Sm/Rif metabolic drift combination this also applies to quinolones (*Linde & associates*, 1993). These markers are named according to the their pattern against of certain laboratory characteristics. Rtt stands for the reversion (of bile and surfactant sensitivity) to bile and surfactant tolerance, which inhibits the inactivation of the orally-administered vaccine by bile (*Linde*, 1982; *Linde et al.*, 1987). Ssq stands for **s**upers**s**ensitivity to **q**uinolones, esp. ciprofloxacin as the most effective antibiotic for combatting salmonellae to date (*Simon & Stille*, 1993; *Linde et al.*, 1993). These outer membrane mutations, which have de facto no influence on parenteral virulence behaviour, function as so-called anti-epidemic markers (reduction of shedding and shortened survival time in the environment = hst, rbt, rtt) and simultaneously as safety and therapeutical markers (supersensitivity to quinolones = ssq).

The 3-marker vaccine strain having the optimum degree of attenuation for chickens was produced by the targeted selection of metabolic drift mutants attenuated to a moderate or low degree and then coupling two such minus-mutants in a series of steps. In addition, the outer membrane mutation (either rtt or ssq) was inserted (*Linde*, 1981; *Linde*, 1983; *Linde et al.*, 1990). This was followed by selecting the predominant serotype from each of the O-groups B, C, D and E (Table 2).

Figure 1: Shedding frequency (percentage of positive samples following enrichment) and duration (last found positive culture, detection limit of approximately 10 bacteria/g faeces) of the vaccine strain S. tm. Nal 2/Rif 9 with (Æ —) and without (• ——) the Rtt marker following single oral vaccination of chicks no older than 36 hours with 10^9 CFU and with S. tm. Nal2/Rif9 (individual data are not recorded, (——)) on the fourth day after hatching (mean values from 3 to 5 tests).

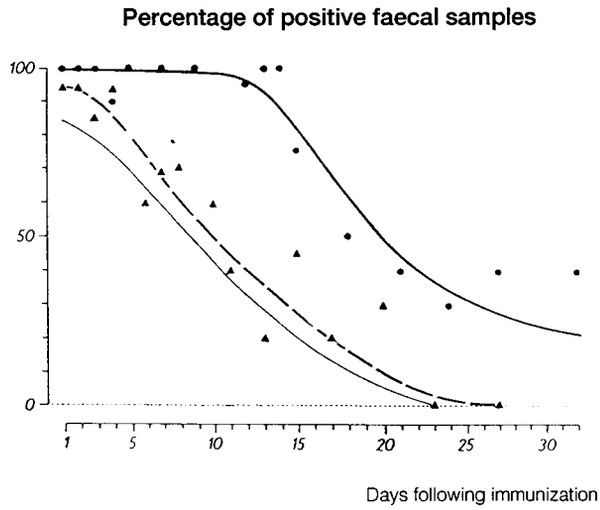


Table 2: S. tm. S. typhi without and with anti-epidemic markers: average survival time in days of 10^6 bacteria/ml of tap water

10^6 bacteria: survival time in days in/of								
tap	S. tm.				S. typhi			
water Ø	Ø	Hst	Rbt	Rtt	Ø	Hst	Rbt	Rtt
	20	8	10	12	7	2	3	4

Vaccine stability in practice

Due to their three separate and independent mutations, the introduced vaccine strains are absolutely stable (in terms of statistical probability) against any risk of back mutation to the wild type strain under practice conditions. Since the total stability of all three markers can be expressed as the product of the stabilities of the individual markers)[at least 10^{-8} (Kaplan, 1969)], the whole stability can be calculated to be at least 10^{-24}. According to the recommendations released by ZKBS (*ZKBS*, 1995), this vaccine has been assigned to risk group I (safe for use as a recipient organism in genetic engineering).

Another note concerning vaccine stability: faster-growing mutants may appear sporadically within vaccine populations whose generation time has been lengthened by 10 minutes. The correlation between lengthened generation time and degree of attenuation that exists for metabolic drift attenuation does not apply when this principle is reversed. These clones having generation times between those of the vaccine strain and the wild type strain not only possess their original, unaltered attenuation markers but also have an additional supressor mutation which merely acts as a means of optimizing the generation time. This has no measurable effect on the degree of attenuation, although it may rise to a level of hyperattenuation. Since the suppressor mutation as a solely mutation also results in a reduction of virulence, these clinically safe clones are actually ideal vaccine strains whose stability is further enhanced by the suppressor mutation (*Linde*, 1993).

Diagnostic differentiation between vaccine and wild type strains

The inserted markers make it quite easy to differentiate between vaccine strains and wild type strains. Due to their metabolic drift (resistance) markers, the vaccine strains grow on culture media with rifampicin and nalidixic acid or with streptomycin (depending on the marker used) and show no growth on media with erythromycin. Wild type strains lacking these genetic alterations exhibit respective growth patterns that are exactly the opposite.

In this respect one limitation should be mentioned. Due to the influence of the anti-epidemic markers, the vaccine strains do not grow on all selective culture media generally used in salmonella diagnostics. However, their growth is not inhibited by some culture media employed in veterinary medicine, such as BPLS, Gassner and XLD agars.

Testing the vaccine strains optimally attenuated for chickens for safe use and efficacy.

Materials and methods

Vaccine strain:
Lyophilised S. tm culture
Nal2/Rif9/Rtt
i.p. LD_{50} (mouse) - 10^6 CFU
GT ~ 32 min. (see Table 3 for additional specifications)

Table 3: Additional laboratory parameters - besides the generation time lengthened from 21-22 min to 31-32 min (as an attenuation equivalent) - of Salmonella typhimurium (tm), enteritidis (ent), infantis (inf) and anatum (ana)

Vaccine strain	w/o nox	Nal20	SM200	Rif200	Ery15	SDO5,000	SDS5,000
S. tm. Nal 2/Rif9/Rtt	100	80	Ø	100	Ø	50	50
S. tm. Ssq/Sm60/Rif42	100	Ø	100	100	Ø	100	60
S. ent. Ssq/Sm24/Rif12	100	Ø	100	100	Ø	80	80
S. inf. Ssq/Sm153/Rif7	100	Ø	100	100	Ø	80	80
S. ana. Ssq/Sm81/Rif	100	Ø	100	100	Ø	90	50
Wild type strains	100	Ø	Ø	Ø	100	100	100

Grow on rates in % on nutrient agar without noxæ (W/O nox) and nutrient agar with µg noxæ/ml. Approx. 100 CFU were swabbed on each plate. (Nal = nalidixic acid; SM = streptomycin; Rif = rifampicin; Ery = Erythromycin; SDO = sodium desoxycholate; SDS = Sodium dodecylsulfate Cultured at 37°C (SDO and SDS) and 43°C (antibiotics).

Wild type (challenge) strains
- S. tm. K 81/92 i.p. LD_{50} (mouse) ~ 10^1 CFU
- S. tm. K 284/93 with a neutral nalidixic acid resistance marker (GT ~ 22 min., like wild type strains)

Culture medium:
Enrichment medium: tetrathionate
BPLS only
BPLS with 20 µg of nalidixic acid and 100 µg of rifampicin/ml, for the selective culture of the vaccine strain

BPLS with 100 µg of nalidixic acid, for the selective culture of the challenge strain

Experimental livestock and keeping:

Calves (fattening breed), weight: 55-64 kg
Hybrid piglets, weight: 25 kg
SPF chicks, Valo
Laying hens (Lohmann Brown)
Broilers (Lohmann Meat)
Test infection in insulating lockers

Experimental materials:

Cloacal swabs
Caecum and -contents
Liver (in toto)
Spleen (in toto)
homogenised and diluted to 1:3, 0.1 ml swabbed on BPLS.
Eggs (shell, egg white and yolk), -cupule or 1 ml

Immunization:

Calves: oral, with milk 10^7 CFU
Piglets: oral, applied to base of tongue with plastic syringe 10^8 CFU
Chicks: oral (cropper instillation) 10^8 to 10^9 CFU
Laying hens/broiler breeding flocks: oral, in drinking water or s.c. 10^8 to 10^{10} CFU

Clinical testing of the S. tm. vaccine strain and field trial

- Test for innocuousity
  - For piglets and calves: monitoring of body temperature, intake of feed, general health and shedding. Test of vaccine strain persistence in the lymph nodes of the duodenum, spleen, kidneys, liver, gall bladder, lungs and mediastinum, as well in the tonsils 14 days after immunization.
  - Stool analysis to detect any presence of the vaccine strain among laboratory, vaccination and farm staff.
  - Tolerance of the 10-fold oral vaccine dose in day-old chicks.
- Shedding period of the vaccine strain in chicks and chickens, length of persistence in internal organs and its transmission to non-vaccinated chickens with immediate contact to vaccinated ones.
  - Shedding and organic persistence in broiler parent stocks.
  - Shedding and contact infection of non-vaccinated chicks in the the same shed.
- Stimulation of systemic immunity following oral vaccination
  - Wild type strain elimination from the liver of orally vaccinated broiler parent stocks in a shorter time than in non-vaccinated control chicks
  - Lower mortality rates following parenteral ~LD85 toxo-infektion and reduced reisolation of the wild type strain from the liver and spleen of chicks given a single oral dose as compared with non-vaccinated control chicks.
- Stimulation of local immunity identified by demonstrating reduced shedding of the wild type strain following oral challenge and in the field trial with large scale flock immunization
  - Reduced shedding of the wild type strain in orally vaccinated broiler parent stocks
  - Gradual eradication of homologous (S. tm.) and partially heterologous (S. e.) salmonella serotypes in the vaccinated chicken flocks

Table 4: Differentiation between TAD Salmonella vac T and S. tm. field strains

Antibiotic	µg/ml	TAD Salmonella vac T (S. tm. Nal2/Rif9/Rtt)	TAD Salmonella vac E (S. e. Ssq/Sm24/Rif12)	field strains
Nalidixic acid	20	resistant	sensitive	sensitive
Streptomycin	100	sensitive	resistant	sensitive
Rifampicin	100	resistant	resistant	sensitive
Erythromycin	15	sensitive	sensitive	resistant

Results

Testing the S. tm vaccine strain for safety and tolerance: tests carried out on piglets and calves

Prior to the start of the field trails the tolerance, persistence and shedding of the specially-developed S. tm. vaccine for chickens was tested on piglets and calves. In particular, the results of testing on calves — whose susceptibility to S. tm is apparently the same as in humans — were expected to provide valuable inferences concerning human safety.

In this test four calves and four weaned piglets were vaccinated orally with 10^7 and 10^8 CFU, respectively. Over the course of the 14-day observation period they exhibited no intolerant reactions, such as fever, diarrhoea, loss of appetite. Daily rectal swabs trailed for isolation of the vaccine strain. Bacteriological tests conducted at the end of the experiment also failed to isolate the vaccine strain in any of the organic samples taken.

No signs of the vaccine strain being transmitted to or colonizing in humans

In the course of laboratory and field trials, stool samples of the laboratory, vaccination and farm staff were examined at regular intervals in order to investigate any possible risks to humans. No salmonellae were detected in these samples.

Testing the tolerance of the 10-fold oral vaccine dose in day-old chicks

Chicks no older than 36 hours were orally administered the prescribed vaccine dose having a 10-fold live bacterial count (max. 10^9 CFU). They showed the same response as the non-vaccinated control chicks in terms of general health, weight increase and mortality.

Shedding period of the vaccine strain, duration of its persistence in internal organs and its transmission to non-vaccinated control chickens in the same stable

- *Shedding in laying hens and detection in the egg*
  Table 5 shows that the vaccination of already egg laying hens results in a shedding of the vaccine strain up to a maximum of 14 days post imm. In a bacteriological test of 273 eggs the vaccine strain was isolated from the shell — but not from the yolk or egg white — of only 10 eggs in the first 7 days post imm. The positive eggs were laid by hens that had been vaccinated with at least 5 x 10^9 CFU.

- *Persistence of the vaccine strain in broiler parent stocks*
  Table 6 shows the results of vaccine strain persistence in 9,493 broilers vaccinated in the field. Samples of liver, spleen (oviducts in laying hens) as well as of the caecum and its contents were taken twice weekly from deceased chickens. Collective samples consisting of no more than 5 individual samples were subjected to bacteriological tests. The approximately three-week

Table 5: Shedding of vaccine strain S. tm. Nal2/Rif9/Rtt by laying hens, including its period of detection on egg shells

after.....days	Investigation of cloacal swabs N	% positive	egg shells N	% positive
2	70	7	67	11.9
7	70	15	139	2.8
14	70	2.8	48	0
21	70	0	19	0

After single vaccination:
time - 20 weeks after hatching
dose- 5 x 10^8 CFU/hen to 1 x 10^{10} CFU/hen oral/s.c.

Table 6: Persistence of the vaccine strain S. tm. Nal2/Rif9/Rtt in broilers

after .... week(s)	Investigation of appendix N	% positive	internal organs N	% positive
1	59	6.7	59	8.4
2	25	4	25	4
3	17	0	17	5.8
4-7	79	0	79	0

Table 7: Shedding and transmission of the vaccine strain S. tm Nal2/Rif/Rtt to hen-house contact chicks

after .....days	Vaccine strain detected in the cloacal swabs of 20 chicks 10^9 CFU oral N	% positive	10 chicks contact chicks N	% positive	20 chicks 10^{10} CFU oral N	% positive	10 chicks contact chicks N	% positive
2	5	25	1	10	11	55	3	30
5	14	70	2	20	14	70	4	40
7	11	55	1	10	1	5	2	20
11	2	10	1	10	1	5	0	0
14	0	0	1	10	0	0	0	0
21	0	0	0	0	0	0	0	0

Oral vaccination ≤ 36 hours after hatching

Table 8: Identification of the wild type strain in the liver of broilers administered 3 oral vaccinations in the rearing phase compared with the non-vaccinated control group

Day post inf.	Group	Chicken no.	Quantitative (log CFU/g liver)	Qualitative (pos/n)
two	vaccinated	1	Ø	
		2	1.5	2/3
		3	Ø	
	control	1	1.5	
		2	2.3	3/3
		3	1.0	
five	vaccinated	1	Ø	
		2	Ø	0/3
		3	Ø	
	control	1	1.2	
		2	1.4	3/3
		3	1.5	
eight	vaccinated	1	Ø	
		2	Ø	0/3
		3		
	control	1	Ø	
		2	1.1	2/3
		3	1.3	
twelve	vaccinated	—	not tested	0/15
	control	—		6/15

persistence is evidence of optimum attenuation and suggests that a good immune response can be expected.

- *Shedding and contact infection among non-vaccinated chicks in the same stable*
  As shown in Table 7, non-vaccinated chicks in the same stable can be infected by the vaccine strain. However, the chicks infected through contact shed the vaccine strain within a few days and only during the shedding period of approximately 14 days exhibited by the vaccinated chicks.

In the course of testing the vaccine strain S. tm. Nal2/Rif9/Rtt comprehensive experiments were conducted to detect the stimulation of systemic and local immunity following oral vaccination (Hahn & associates, 1993; Vielitz & Hahn, 1994).

Stimulation of systemic immunity following oral immunization

Quicker elimination of the wild type strain from the liver of broilers orally vaccinated at the ages of 1 day, 7 days and 15 weeks than in non-vaccinated control chicks.
Table 8 shows that the wild type strain orally administered in the 50th week after hatching accumulates in the internal organs of vaccinated chickens with significantly lower bacterial counts than in non-vaccinated chickens. In addition, the wild type strain was eliminated within five days. Persistence of the wild type strain could be identified in all non-vaccinated chickens after five days, and in approximately 40% of them after 12 days.
Mortality and elimination of the wild type strain within 14 days from the liver and spleen in chicks orally vaccinated with 10^8 CFU one day after hatching as well as in control chicks; both groups being given a parenteral LD_{85} toxoinfection (5×10^8 CFU) at the age of 10 weeks
The data in Table 9 show that a distinct systemic immunity is still present 10 weeks after the single vaccination of day-old chicks as demonstrated by the drastic reduction in mortality rates following toxoinfection compared to the control chickens and the more rapid elimination of the wild type strain in the surviving chickens.

Table 9: Systemic immunity following single oral vaccination

	Number	Mortality (%)	Wild type strain in internal organs
Vaccinated	18	2/18 (11)	0/5
Control	20	17/20 (85)	3/3

Stimulation of a local immunity that reduces wild type strain shedding following oral administration

Reduction of wild type strain shedding in adult broiler parent stocks which were orally vaccinated with a dose of 1×10^8 as day-old chicks, followed by two additional vaccinations in the 7th and 15th week. In the 50th week of age they were orally infected with 1.8×10^{10} CFU of the wild type strain.
The test results listed in Table 10 show greatly reduced shedding of the wild type strain by orally vaccinated chickens which were subsequently given a massive oral infection.

Table 10: Wild type strain shedding by broilers orally vaccinated with the vaccine strain S. tm. Nal2/Rif9/Rtt

after ... days	Wild type strain detection in cloacal swabs of vaccinated chickens N	% positive	control group N	% positive
2	24	66.6	24	83.3
5	21	52.4	21	66.6
8	18	22.2	18	61.1
12	15	0	15	33.3

Gradual eradication of homologous and partially heterologous salmonella serotypes in chicken flocks vaccinated on a large scale

Large-scale field studies conducted on 750,000 chickens tested the live S. typhimurium vaccine TAD Salmonella vac T with the following vaccination programme:

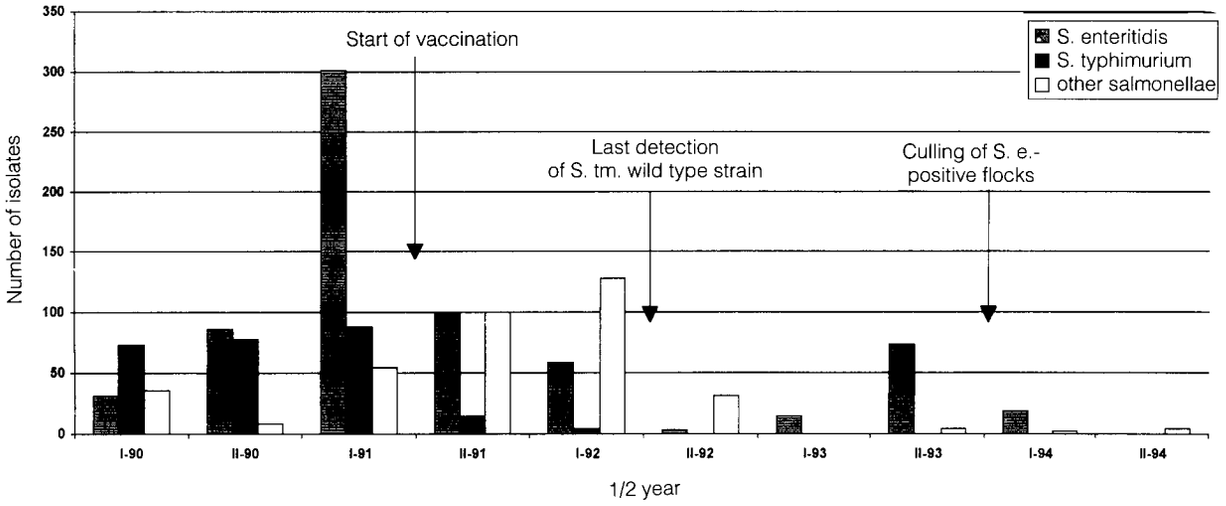
Laying hens: oral: 1st day after hatching, 7th and 16th week after hatching
Breeders: oral: 1st day after hatching, 7th week after hatching
subcutaneous: between the 16th and 18th week after hatching

The vaccine was also used at a breeding farm starting in the summer of 1991. 200,000-250,000 breeders (layers and broilers) in 50 hen houses were vaccinated annually. An extensive bacteriological monitoring programme was strictly followed, which included meconium sampling as well as the examination of deceased chickens.

Bacteriological tests started on the first day after hatching with the taking of meconium and chick-plug samples. Losses were examined each week, with trailer and faecal examinations being conducted on a monthly basis. A total of 10,000-14,000 collective samples were tested each year, of which approximately 20% were taken from hatcheries and 80% from flocks. Rodents, wild type birds and beetles were included in the tests whenever possible.

The detection rates of S. tm. and S.e. dropped after the vaccination programme was introduced (Fig. 2). In the summer of 1992 S.tm. was no longer detected, which can be attributed to the effective homologous immunization of the vaccine employed. The II/94 S. tm. isolate came from a gull. The detection rate of S. e. also dropped dramatically due to the effects of partial cross immunity. Whereas 80 S. e. isolates were detected monthly before the vaccine programme was initiated, only sporadic cases of isolates occurred in the course of 1992/93. At the end of 1993 there was only an outbreak of S. e. in three hen-houses. Two flocks were kept in floor management, one in cages. The source of infection must have been the feed, because the infection occurs at the same time. Since salmonella shedding persisted despite metaphylactic measures — antibiotics followed by competitive exclusive flora — these chickens were prematurely slaughtered in the first quarter of 1994. Except for two cases of II/94 in mice, no further S. e. isolates were found.

Figure 2: Salmonella isolates 1990-1994 in vaccinated German chicken flocks



Discussion of the results

In the course of developing the S. tm live vaccine TAD Salmonella vac T extensive safety and tolerance tests were carried out on the target chick/chicken species. Even the 10-fold oral vaccine dose proved to be well tolerated by day-old chicks. No deaths or physical disorders resulted from the immunization. There were no noticeable aberrations in feeding habits or weight increase.

In order to establish that the mutants pose no threat to human health, calves — whose susceptibility to S. tm is apparently the same as in humans — and weaned piglets were also tested. This also confirmed acceptable tolerance at the tested dosage levels of 10^7 CFU (calf) and 10^8 (piglet). At the end of the testing period (14 days post imm.) the vaccine strain could not be reisolated from any of the tested organs. Other indications that with proper handling the vaccine strain cannot be transmitted to or colonized by human were controlled by regular stool analyses of the vaccine and farm personnel during the controlled field studies. The vaccine strain was not detected in a single case. These findings suggest that the chance of endangering human health is very improbable.

Other experiments were made to test the shedding period, length of persistence in internal organs and the transmission of the vaccine strain to non-vaccinated contact chickens in the same stable. The vaccine strain tested here, which carries a marker that reduces shedding, was no longer detected than 14 days. To ensure an adequate margin of safety, a 21-day waiting period is prescribed between vaccination and slaughtering/delivery of eggs. Care should be taken that immunization is conducted during rearing and concluded in the 16th to 18th week after hatching. This adequately ensures that no transmission to humans can occur.

The markers inserted in the antibiogram make it quite easy to separate vaccine strains from wild type strains and this can be done in any bacteriological laboratory.

The potency of the S. tm. vaccine strain specially developed for chickens was determined by demonstrating the stimulation of systemic and local immunity after oral vaccination (in part with s.c. boosters). Tests of immunization and challange showed that the wild type strains in the internal organs of orally vaccinated chicks and chickens accumulated at lower bacterial counts and were eliminated faster. Furthermore, compared with non-vaccinated chickens, the vaccinated group shed the bacterial strain in a shorter period of time and with reduced germ numbers. The fact that vaccinated chickens did not shed any more wild type strains after 12 days whereas in non-vaccinated controls the shedding exceeds over this time limit. This suggests that the chain of infection can be broken in vaccinated flocks by lowering the infection load (remaining below the minimum infective dose). These effects were achieved in large-scale field studies. After the introduction of the immunization programme where TAD salmonella vac T was administered on the first day, 7th and 16th week after hatching, latent S. tm. infections disappeared in the vaccinated flocks. The anticipated partial cross immunity resulted in a significant reduction of S. e. findings.

The vaccination programme was also accompanied by comprehensive hygienic measures. The use of the S. e. live vaccine (which is still in the testing phase) in the planned S. tm/S. e. bivalent vaccine will make it possible to eradicate[1] latent salmonella infections in chicken flocks and thereby better protect humans from salmonella-related enteritis (including lethal cases) transmitted by eggs and chicken meat.

[1] Eradicated in the sense that salmonellosis in vaccinated flocks remains below the detection limit or that salmonella infection caused by external vectors can be eliminated within a short time by the immunized flocks.

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