



## Possible associations between *Salmonella* persistence in poultry houses and resistance to commonly used disinfectants and a putative role of *mar*

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### Abstract

A putative link between *Salmonella* persistence in the agricultural sector and resistance to disinfectants has been sparsely investigated. Therefore, minimum inhibitory concentration (MIC) tests against five disinfectants commonly used in poultry premises (formaldehyde, glutaraldehyde/benzalkonium chloride compound, oxidising compound, tar oil phenol, iodophor) were performed on 286 *Salmonella* isolates, including 256 from Danish broiler houses, altogether representing nine serotypes. Six of these isolates were used for adaptation and de-adaptation studies involving the five disinfectants. Amongst 60 of these isolates selected for growth studies in cyclohexane (possibly associated with up-regulated efflux), only one isolate grew. From this isolate and the six isolates used in the adaptation and de-adaptation studies, mutants highly resistant to triclosan (a disinfectant linked with *mar*-type resistance) were selected. In addition, adaptation and de-adaptation studies with triclosan were performed.

For the 286 isolates, the small variations in MICs could not be associated with *Salmonella* persistence in Danish broiler houses or previous use of relevant disinfectants. Adaptation and de-adaptation did not alter MICs to the five farm disinfectants. Compared to the parent isolates, MICs for the triclosan adapted and de-adapted isolates and the triclosan mutants were significantly increased to triclosan, but not to the five disinfectants. Moreover, most of the triclosan adapted and de-adapted isolates grew in cyclohexane. Thus, there was no correlation between triclosan and cyclohexane resistance on one hand and resistance to the five disinfectants on the other, suggesting that triclosan resistance is not linked with resistance to these disinfectants.

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### 1. Introduction

Since 1992, samples for *Salmonella* examination have been submitted from all Danish broiler flocks

when the chickens are ca. 3 weeks old (Bisgaard, 1992; Anon., 2003). Data from each flock, including the use of disinfectants in each download period, have been recorded electronically in the ante mortem (AM) database (Danish Poultry Council) during the same period (Angen et al., 1996). In general, *Salmonella* Enteritidis, *Salmonella* Typhimurium and *Salmonella* Tennessee were introduced to the broiler houses with the day-old chicks (Christensen et al., 1997; Gradel and Rattenborg, 2003), whereas the sources of other serotypes (mainly *S. Infantis*, *S. 4.12:b:-* and *S. Indiana*) were more difficult to trace (Gradel and Rattenborg, 2003). In the last 5 years, 0.2–3.1% of the broiler flocks have been *Salmonella*-positive (Anon., 2001, 2003). A few farms persistently infected with *S. Infantis*, *S. 4.12:b:-* or *S. Indiana* have mainly contributed to this *Salmonella* occurrence, whereas the hatchery-introduced serotypes have usually been eliminated within one or a small number of flock cycles (Gradel and Rattenborg, 2003). The reasons for these differences in tendencies to persist have never been elucidated. Repeated use of the same types of antibiotics is known to favour the development of antibiotic resistance, but less is known about the use of disinfectants. A few disinfectant types are used commonly in the Danish poultry sector, and on many farms, the same disinfectant brand is often used for years in all download periods in all broiler houses. Theoretically, this could induce resistance against the disinfectant used, and this could – at least partly – explain the persistence of *Salmonella*. Most other disinfectant resistance studies have been from hospital wards or the food industry, but few studies have addressed this topic in animal houses where other disinfectant types are often used (Linton et al., 1987; Strauch and Böhm, 2002).

In recent years, many studies have dealt with multiple antibiotic resistance mediated by up-regulation of efflux (e.g., up-regulation of the AcrAB efflux pump or global regulators such as *marRAB* and *soxRS*) in connection with antibiotic and disinfectant resistance (Levy, 2002; Randall and Woodward, 2002), but little has been published on the role of these types of efflux mechanisms in resistance to disinfectants commonly used in the agricultural sector.

Thus, there were several aims of this study. Minimum inhibitory concentration (MIC) studies involving five disinfectants commonly used in the

poultry sector were performed for six *Salmonella* serotypes to see if MICs could be related to *Salmonella* persistence or use of disinfectants in Danish broiler houses. *Salmonella* from other sources were included in the MIC studies to obtain a broader epidemiological perspective. In addition, six isolates, three with high and three with low MICs to the five disinfectants, were used in adaptation and de-adaptation studies involving the same five disinfectants to see if resistance could be introduced and maintained in the laboratory. Finally, triclosan resistant mutants were selected from some isolates, and these mutants were tested for growth in cyclohexane and resistance to the five disinfectants to determine if there was some shared efflux type resistance mechanism.

## 2. Materials and methods

### 2.1. *Salmonella* from Danish broiler houses

During the study period (January 1992–October 2001), Denmark had about 360 broiler premises with a total of 760 broiler houses (range 1–16 houses per premise) (AM database, Danish Poultry Council). In all broiler houses, an all-in all-out system was applied and cleaning and disinfection was performed in all download periods (i.e., when there were no broilers in the houses). The use of disinfectants, up till three types per flock, was recorded for each download period (AM database, Danish Poultry Council). Usually, a period of 5–6 weeks elapsed from insertion of the day-old chicks until sending the broilers to the abattoir, followed by a 8–20-day long download period, i.e., 6–7 broiler flocks were raised annually in each house (AM database, Danish Poultry Council) (Angen et al., 1996).

From nearly all broiler houses included in this study, two or more isolates were selected, representing both the beginning and the end of the persistence period (Table 1). For houses with two or more isolates, periods between isolates (cf. Table 1) were used to calculate if MICs changed during the period in which the serotype persisted in the broiler house.

### 2.2. Bacterial isolates

Danish *Salmonella* isolates were stored in Standard Count Agar (Merck 1.01621), whereas English isolates

Table 1

Numbers of isolates from Danish broiler houses used in the study and calendar months between first and last isolate (period January 1992–October 2001)

Serotype	Numbers of isolates per house used in the study						Months between first and last isolate in each house			
	1	2	3	4	5	6	Mean	Median	1st–3rd quartile	Range
<i>S. Enteritidis</i>	2 <sup>a</sup>	14		1			11.5	6.0	4.5–11.5	2–47
<i>S. Typhimurium</i>	2	17	1				8.6	5.0	2.0–9.0	1–35
<i>S. Tennessee</i>		12					5.8	4.5	2.8–9.0	1–16
<i>S. 4.12:b:-</i>	2	14	3	5	2	2	36.1	33.0	13.0–58.8	3–80
<i>S. Infantis</i>		22	3	2			20.3	8.0	3.0–28.0	1–101
<i>S. Indiana</i>	3	5		1			9.0	7.0	3.3–9.3	2–26
Total number of houses ( <i>n</i> = 113)	9	84	7	9	2	2				
Total number of isolates ( <i>n</i> = 256)	9	168	21	36	10	12				
Total number of periods <sup>b</sup> ( <i>n</i> = 143)	0	84	14	27	8	10				

<sup>a</sup> Numbers of broiler houses.

<sup>b</sup> Numbers of periods between isolates, calculated as (total number of houses) × (number of isolates per house used in the study – 1).

were stored on Dorset's egg slopes (Med-Ox Diagnostics Inc., EM300), all at room temperature. Three strains of *Escherichia coli* (NCTC 10418, AG100 and AG102) were used as controls in all MIC test batches. All English *Salmonella* isolates, except *S. Choleraesuis* NCTC 10653, were from samples taken from the same sites both before and after disinfection (cf. Table 2) using methods described previously (Davies and Wray, 1994, 1995, 1997; Davies et al., 2001). Because the English *S. Senftenberg* isolates had high MICs, Danish

*S. Senftenberg* isolates were included to see if this applied more generally to this serotype.

### 2.3. Disinfectants used in the MIC tests

There were 4629 *Salmonella*-positive Danish broiler flocks in the period from 3 January 1992 to 2 October 2001 (AM database, Danish Poultry Council). Among these, a glutaraldehyde/benzalkonium chloride compound, formaldehyde and an

Table 2

Bacterial isolates used in the study

Country	Type	Number of isolates	Source and description
DK <sup>a</sup>	<i>S. Enteritidis</i>	34	Danish broiler houses (cf. Table 1)
	<i>S. Typhimurium</i>	39	Danish broiler houses (cf. Table 1)
	<i>S. Tennessee</i>	24	Danish broiler houses (cf. Table 1)
	<i>S. 4.12:b:-</i>	81	Danish broiler houses (cf. Table 1)
	<i>S. Infantis</i>	61	Danish broiler houses (cf. Table 1)
	<i>S. Indiana</i>	17	Danish broiler houses (cf. Table 1)
	<i>S. Senftenberg</i>	13	Danish poultry sector, mainly from breeding houses
UK	<i>S. Choleraesuis</i> NCTC 10653	1	Strain used in the official English DEFRA disinfection tests
	<i>S. Typhimurium</i> , DT104	8	Pig and broiler farms, b/a <sup>b</sup> disinfection with phenol, formaldehyde or peroxygen
	<i>S. 4.12:d:-</i>	4	Feed mill and hatchery, b/a disinfection with formaldehyde
	<i>S. Senftenberg</i>	4	Hatchery, b/a disinfection with formaldehyde, glutaraldehyde or QAC <sup>c</sup>
	<i>E. coli</i> NCTC 10418	1	Control strain
	<i>E. coli</i> AG100	1	Control strain
	<i>E. coli</i> AG102	1	Control strain, <i>mar</i> <sup>d</sup> mutant of <i>E. coli</i> AG100

<sup>a</sup> Denmark.

<sup>b</sup> Before or after.

<sup>c</sup> Quaternary ammonium compound.

<sup>d</sup> Multiple antibiotic resistance regulon which up-regulates the AcrAB efflux pump (cf. text).

oxidising compound were used most commonly for disinfection of broiler houses (38.8, 32.4 and 14.9%, respectively), whereas phenols and iodophors were rarely used (AM database, Danish Poultry Council). In the UK, phenols were used commonly in poultry houses, whereas iodophors were used mainly for water systems, foot dips and general disinfection (Davies and Wray, 1995; Davies et al., 2001). Therefore, the following three “Danish” and two “English” disinfectants were chosen for this study: (1) a glutaraldehyde (23%, v/v) and benzalkonium chloride (5%, v/v) compound (Bio Komplet<sup>®</sup> Plus and the corresponding pH-regulator (KOH and H<sub>3</sub>PO<sub>4</sub>), Korn-og Foderstof Kompagniet, Viby J, Denmark), (2) formalin (24.5%, v/v, formaldehyde, no. 4552, Bie & Berntsen A/S, Rødovre, Denmark), (3) an oxidising compound (Virkon<sup>®</sup> S, Antec International, Sudbury, England), (4) a high boiling tar acid phenol compound (Farm Fluid S<sup>®</sup>, Antec International, Sudbury, England) and (5) an iodophor (FAM 30<sup>®</sup>, Evans Vanodine International, Preston, England).

#### 2.4. MIC tests

MIC tests were performed as previously described (Randall et al., 2001). On the day of performing the MIC tests, pH-regulator was added to Bio Komplet Plus (1:11) according to manufacturer's instructions and disinfectant solutions in sterile deionised water were prepared and used for double dilutions. Ranges of 2–250 µl, 8–1000 mg and 8–1000 µl/100 ml were made for formaldehyde, Virkon S and the remaining disinfectants, respectively. For all *Salmonella* isolates, the tests were performed at least in duplicate on different days. Each batch consisted of one multipoint inoculator plate with 20 wells, 17 with *Salmonella* isolates and 3 with the same *E. coli* control strains used in all batches (to check for deviations between these).

#### 2.5. Disinfectant adaptation and de-adaptation tests

Three isolates with high and three isolates with low MICs to the five disinfectants (cf. Table 8; isolates that did not grow in cyclohexane) were selected and used for the adaptation and de-adaptation tests which were performed in duplicate, each involving one of the five disinfectants. Initially, isolates were grown overnight in

3.0 ml Luria Bertani (LB) broth (Difco 0446) at 37 °C. A 0.1 ml inoculum was passaged to 3.0 ml LB broth with a disinfectant concentration of half the lowest recorded MIC, incubating overnight at 37 °C. Each consecutive day, the disinfectant concentration in LB broth was increased by a factor of 1.5, and a 0.1 ml inoculum from the LB broth grown the previous day was inoculated into this. Turbidity was registered visually and cultures were plated on blood agar (BA) (blood agar base (Oxoid CM271), 5% calf blood) plates to check for growth and purity. The passages ceased when no turbidity and no growth on BA were observed. LB broth (1.5 ml) with growth at the highest disinfectant concentration was transferred to an Eppendorf tube and centrifuged for 5 min at 15,890 × *g*. The pellet was suspended in physiological saline to McFarland 0.5 and used for MIC tests as described above. For de-adaptation, 0.1 ml LB broth was passaged to 3.0 ml LB broth without disinfectant for six consecutive days, after which the MIC tests were repeated.

#### 2.6. Triclosan studies

Strains up-regulated for efflux are likely to show reduced susceptibility to the biocide triclosan (Irgasan DP300, donated by Ciba Specialty Chemicals Denmark, Århus) (Levy, 2002), but little is known about a putative association between efflux type resistance and resistance to the five disinfectants of this study. An amount of 32 mg triclosan was suspended in 5.0 ml sterile deionised water, and 1N NaOH was added drop by drop until all triclosan was dissolved; then, sterile deionised water was added up to 4000 ml, yielding 8 µg/ml triclosan. The six isolates from the adaptation and de-adaptation studies (cf. above) and the one isolate that grew in cyclohexane (cf. below) were used to select isolates for growth at high triclosan concentrations. Agar plates were made with 4.0 µg/ml triclosan. Isolates were grown overnight in 500 ml LB broth at 37 °C. Forty-five millilitres LB broth was centrifuged five times for 20 min at 4388 × *g*, each time discarding the supernatant and adding more broth. Then, 2.5 ml physiologic saline was added to the final pellet and 10-fold dilutions were plated on BA to determine numbers of CFU ml<sup>-1</sup>. For each isolate, 0.1 ml of the undiluted solution was streaked on each of five agar plates containing 4.0 µg/ml triclosan, incubating 42–48 h at 37 °C after which

CFU were counted. For each isolate, one triclosan resistant mutant colony was isolated and used for MIC tests. The parent isolates, their triclosan resistant counterparts and the *E. coli* control strains were used for MIC tests with the five disinfectants and triclosan, performed in duplicate on different days.

For the same seven isolates, adaptation and de-adaptation studies with triclosan were made in duplicate as for the other five disinfectants (see above), beginning with 0.13 µg/ml triclosan (dissolved in NaOH as described above). The adaptation studies were discontinued on day 20, as so much NaOH had to be used for dissolving triclosan for day 21 that pH of the agar became too alkaline. After adaptation, MIC tests were performed in duplicate for the parent and the adapted isolates and the *E. coli* control strains against both the five disinfectants and triclosan (double dilutions in the range 0.03–128.8 µg/ml (pH of the latter was 6.8)). After de-adaptation, only triclosan was used in the MIC tests, as no changes were seen for the other five disinfectants after adaptation, and the parent isolates were omitted in these studies.

### 2.7. Cyclohexane resistance tests

Strains with up-regulation of efflux (e.g., up-regulation of the AcrAB efflux pump or global regulators such as *marRAB* and *soxRS*) have been shown to be cyclohexane resistant (White et al., 1997). As such, cyclohexane resistance is a useful marker for strains with up-regulated efflux (Randall et al., 2001), and such strains may show resistance to unrelated antibiotics and disinfectants. For this reason, 60/286 isolates, representing high MICs against the five disinfectants (cf. above), were selected for cyclohexane resistance tests as described by Asako et al. (1997). In addition, cyclohexane resistance tests, all in duplicate, were performed for isolates adapted/de-adapted to/from triclosan and triclosan mutant isolates.

### 2.8. Statistical analysis

All data were entered in an Access database (Anon., 1997).

For each disinfectant, the MICs for all *Salmonella* serotypes were compared by the non-parametric Kruskal–Wallis one-way analysis of variance (Anon., 2002). This was also done within each disinfectant for

the three *E. coli* control strains used in all batches. For significant bacteria/disinfectant combinations, the MICs between all pairs of isolates were compared using Dunn's method (Anon., 2002), as this method sets an error level for the whole set of comparisons, thus avoiding too many 'significant' differences due to the many comparisons.

The MICs were merged (formaldehyde: 4/8 versus 15/30; Bio Komplet Plus: 60 versus 125/250; Virkon S: 60/125 versus 250; Farm Fluid S: 15/30 versus 60/125; FAM 30: 60/125 versus 250/500) to form 2 × 2 tables for pair wise comparisons by chi-square tests, or if expected values were <5, two-tailed Fisher exact tests. These merged categories and the same tests were also used to assess whether the MICs were related to using or not using a "Danish" disinfectant in the preceding download period. The periods between isolates in all broiler houses with ≥2 isolates (cf. Table 1) were used to calculate putative MIC changes for the five disinfectants compared by the Kruskal–Wallis test.

A significance level of 5% ( $p = 0.05$ ) was used in all relevant tests.

## 3. Results

### 3.1. MIC tests

For both *Salmonella* and *E. coli* and for all disinfectants, the Kruskal–Wallis analysis was significant, except for *E. coli* and Farm Fluid Super (Table 4).

Among the isolates from Danish broiler houses, multiple comparisons generally showed that *S. Tennessee* had significantly higher MICs to formaldehyde, Virkon S and FAM 30, *S. 4.12:b:-* had to Bio Komplet Plus, Virkon S and FAM 30 and *S. Infantis* had to Virkon S and FAM 30 (Table 4). Thus, higher MICs were found for several serotypes regardless of their tendency to persist in broiler houses (cf. Table 1), and also included "English" disinfectants.

Among isolates not coming from Danish broiler houses, *S. Senftenberg*, both from DK and the UK, had high MICs to formaldehyde, and the English *S. Senftenberg* had high MICs to Virkon S and FAM 30 (Tables 3 and 4). Among the *S. Typhimurium* DT104 isolates, there were generally few deviations from the general distribution although five isolates were resistant to at least six types of antibiotics (data not

Table 3  
Microbial inhibitory concentrations of isolates

Country	Type	MIC <sup>a</sup>																			
		Formaldehyde				Bio Komplet Plus					Virkon S				Farm Fluid S				FAM 30		
		4	8	15	30	15	30	60	125	250	30	60	125	250	15	30	60	125	60	125	250
DK <sup>b</sup>	<i>S. Enteritidis</i>	34 <sup>c</sup>				14 20					6 22 6 1				12 21				4 9 21		
	<i>S. Typhimurium</i>	39				20 17 2					12 24 3				19 20				7 15 17		
	<i>S. Tennessee</i>	9 8		7		6 16 2					1 7 16				4 20				2 22		
	<i>S. 4.12:b:-</i>	66		15		12 68 1					1 58 22				17 64				1 13 66 1		
	<i>S. Infantis</i>	1 60				18 43					6 37 18				29 31 1				17 37 7		
	<i>S. Indiana</i>	16		1		11 6					7 9 1				16 1				14 3		
	<i>S. Senftenberg</i>			12 1		5 8					10 3				1 12				10 3		
UK	<i>S. Choleraesuis</i> NCTC 10653	1				1					1				1				1		
	<i>S. Typhimurium</i> , DT104	8				7 1					8				8				2 6		
	<i>S. 4.12:d:-</i>	4				4					4				4				4		
	<i>S. Senftenberg</i>			4		4					4				4				4		
	<i>E. coli</i> NCTC 10418	1 30		12 17							1 19				11 2 26 4				6 25 1		
	<i>E. coli</i> AG100	8 23		5 24		1					3 13 14				6 25 1				12 17 1		
	<i>E. coli</i> AG102	11 20				26 4					1 13 16				2 28 1				6 23 1		

<sup>a</sup>  $\mu$ l/100 ml, except mg/100 ml for Virkon S.

<sup>b</sup> Denmark.

<sup>c</sup> Number of isolates/batches for *Salmonella/E. coli*, respectively.

shown). The MICs of all the English isolates were generally the same before and after disinfection (Table 3). MICs for *S. Choleraesuis* NCTC 10653 did not deviate significantly for any of the five disinfectants, indicating it is representative for *Salmonella* in the official UK DEFRA disinfection tests.

For formaldehyde, most isolates differed from the isolates having high MICs, whereas for the other four disinfectants, fewer isolates differed from the high MIC isolates (range 1–3, except for the UK *S. Senftenberg* and FAM 30) (Table 4).

Among the *E. coli* control strains, AG100 and AG102 generally had similar MICs (Tables 3 and 4), so *mar* did not seem to play a significant role. Compared to these two isolates, *E. coli* NCTC 10418 had significantly higher (formaldehyde, Virkon S and FAM 30) or lower (Bio Komplet Plus) MICs (Table 4).

Disinfectants were compared pair wise (MICs merged, see above) to deduce putative associations for isolates (Table 5). In general, there were strong associations between MICs for different disinfectants, except for formaldehyde versus Bio Komplet Plus, Virkon S and FAM 30, respectively.

For the 104 broiler houses with  $\geq 2$  isolates (Table 1), increases and decreases in MICs during the persistence period were recorded (Table 6). For all

five disinfectants merged, there were 98 increases, 529 periods without MIC changes and 88 decreases ( $p = 0.38$ ), i.e., there were no significant MIC changes.

For the three “Danish” disinfectants (formaldehyde, Bio Komplet Plus, Virkon S), cross-tabulations between the use of such compounds in the preceding download period and merged MICs (see above) were made, but no significant differences in MICs were seen between flocks using or not using the actual disinfectant with regard to both “Danish” and “English” disinfectants (Table 7). The lowest  $p$ -values were found for Virkon S related to the use of glutaraldehyde/quaternary ammonium compound ( $p = 0.07$ ) or an oxidising compound ( $p = 0.08$ ) in the preceding download period, albeit MICs tended to be lower, and not higher, if an oxidising compound had been used.

### 3.2. Disinfectant adaptation and de-adaptation tests

In LB broth, growth ceased at concentrations up to ca.  $13 \times$  MIC (highest range for formaldehyde (5.5–12.5) and Virkon S (3–12.5), lowest range (0.7–4.2) for Bio Komplet Plus) (data not shown). This was,



Table 4  
Statistical comparisons between MICs of isolates

Disinfectant	Kruskal–Wallis overall <i>p</i>	Individual differences ( <i>p</i> < 0.05)
<i>Salmonella</i>		
Formaldehyde	<0.001	DK/Ten > DK/Ent, DK/Typ, DK/4.12:b:-, DK/Inf, DK/Ind, UK/DT104 <sup>a</sup> DK/Sen > DK/Ent, DK/Typ, DK/4.12:b:-, DK/Inf, DK/Ind, UK/DT104, UK/4.12:d:- UK/Sen > DK/Ent, DK/Typ, DK/4.12:b:-, DK/Inf, DK/Ind, UK/DT104, UK/4.12:d:-
Bio Komplet Plus	<0.001	DK/4.12:b:- > DK/Typ, DK/Ind UK/DT104 > DK/Ind
Virkon S	<0.001	DK/Ten > DK/Ent, DK/Typ, DK/Ind DK/4.12:b:- > DK/Typ, DK/Ind DK/Inf > DK/Typ UK/Sen > DK/Typ, DK/Ind
Farm Fluid S	<0.001	DK/Ind < DK/Ent, DK/Ten, DK/4.12:b:-, DK/Inf, DK/Sen, UK/DT104, UK/4.12:d:-, UK/Sen
FAM 30	<0.001	DK/Ten > DK/Typ, DK/Ind, DK/Sen DK/4.12:b:- > DK/Typ, DK/Ind, DK/Sen DK/Inf > DK/Typ, DK/Ind, DK/Sen UK/Sen > DK/Ent, DK/Typ, DK/Inf, DK/Ind, DK/Sen
<i>E. coli</i>		
Formaldehyde	0.007	NCTC > AG102
Bio Komplet Plus	<0.001	AG102 > NCTC AG100 > NCTC
Virkon S	<0.001	NCTC > AG100, AG102
Farm Fluid S	0.102	–
FAM 30	<0.001	NCTC > AG100, AG102

<sup>a</sup>  $x > y/x < y$ : *x* has significantly higher/lower MICs than *y*. Designations for *Salmonella*: country/first three letters (except 4.12:b:- (*S.* 4.12:b:-), 4.12:d:- (*S.* 4.12:d:-) and DT104 (*S.* Typhimurium, DT104)). Designations for *E. coli*: NCTC = NCTC 10418. Countries and bacteria types, cf. Table 3.

however, not reflected in similarly high MICs after adaptation, where all isolates, except one, were within one double dilution compared to the MICs of the parent isolates (data not shown). Four of the six

isolates adapted to Virkon S grew weakly in the last LB broth before growth ceased, so a pellet big enough to obtain McFarland 0.5 could not be obtained, and growth could not be re-established in the six

Table 5  
Minimum inhibitory concentrations (MICs) of the 286 *Salmonella* isolates—2 × 2 tables related to disinfectants

		Bio Komplet Plus		Virkon S		Farm Fluid S		FAM 30	
		(60) <sup>a</sup>	(125/250)	(60/125)	(250)	(15/30)	(60/125)	(60/125)	(250/500)
Formaldehyde	(4/8)	77 <sup>b</sup>	161	182	56	93a	145a	81	157
	(15/30)	9	39	31	17	7a	41a	14	34
Bio Komplet Plus	(60)			81b	5b	70b	16b	65b	21b
	(125/250)			132b	68b	30b	170b	30b	170b
Virkon S	(60/125)					96b	117b	95b	118b
	(250)					4b	69b	0b	73b
Farm Fluid S	(15/30)							72b	28b
	(60/125)							23b	163b

<sup>a</sup> All numbers in brackets: MICs (units as in Table 3).

<sup>b</sup> All numbers not in brackets: numbers of isolates (no letters:  $p > 0.05$ ; a:  $p < 0.01$ ; b:  $p < 10^{-6}$ ).

Table 6  
Changes in minimum inhibitory concentrations (MICs) for disinfectants in periods (cf. Table 1)

Disinfectant	MIC increases	No MIC changes	MIC decreases	Sum
Formaldehyde	7	125	11	143
Bio Komplet Plus	19	110	14	143
Virkon S	31	90	22	143
Farm Fluid S	14	110	19	143
FAM 30	27	94	22	143
Sum	98	529	88	715

de-adaptation passages. Moreover, four isolates after adaptation did not grow in the MIC tests with formaldehyde, regardless of which disinfectant they had been adapted to, but all of these could be tested after de-adaptation. After de-adaptation, no changes beyond one double dilution compared to the MICs of the parent isolates were seen (data not shown).

### 3.3. Selection of triclosan resistant mutants

After centrifugation, the concentration range for the seven isolates was  $1.4\text{--}2.0 \times 10^{10}$  CFU ml<sup>-1</sup>. On the five agar plates, 1/1/4/4/15/37/143 CFU grew per

isolate, thus yielding mutation rates in the range from  $1.3 \times 10^{-10}$  to  $2.0 \times 10^{-8}$  (highest for the isolate that grew in cyclohexane) (Table 8).

For the five disinfectants, virtually all changes in MICs for the mutants and adapted isolates were within one double dilution compared to the seven parent isolates (data not shown), whereas there were big increases in MICs for triclosan.

### 3.4. Triclosan adaptation and de-adaptation tests

Most isolates grew until day 20 of the adaptation, as only three replicates ceased their growth before (one on day 1 and two on day 8) (Table 8). In general, the MICs to triclosan after adaptation and de-adaptation correlated well with the number of days they grew, except that the second replicate of isolate 9578243 illogically had a low MIC after adaptation whereas it was high after de-adaptation.

### 3.5. Growth in cyclohexane (parent isolates)

All isolates used in cyclohexane resistance studies grew on agar without organic solvent (data not shown). In nearly all batches, *E. coli* AG102 grew in

Table 7

Use of three disinfectants in the preceding download period versus minimum inhibitory concentrations for *Salmonella* isolated from broiler flocks after the download period<sup>a</sup>

Disinfectant	MIC <sup>b</sup>	Formaldehyde used in preceding period		Glutaraldehyde/QAC <sup>c</sup> used in preceding period		Oxidising compound used in preceding period	
		Yes	No	Yes	No	Yes	No
Formaldehyde	4/8	23 <sup>d</sup>	98	61	58	28	93
	15/30	5	12	6	10	5	12
Bio Komplet Plus	60	8	31	17	20	10	29
	125/250	20	79	50	48	23	76
Virkon S	60/125	21	80	44	54	28	73
	250	7	30	23	14	5	32
Farm Fluid S	15/30	11	41	22	28	9	43
	60/125	17	69	45	40	15	71
FAM 30	60/125	9	31	18	21	8	32
	250/500	19	79	49	47	16	82

<sup>a</sup> There were 138, and not 143 (cf. Table 1), isolates after potential use of formaldehyde or an oxidising compound in the preceding download period, as disinfectants were not recorded for five download periods. After potential use of glutaraldehyde/QAC in the preceding download period, three more download periods, in which only QACs were used, were omitted.

<sup>b</sup> Minimum inhibitory concentration (units as in Table 3).

<sup>c</sup> Quaternary ammonium compounds.

<sup>d</sup> Numbers of isolates.



Table 8

Data for the seven isolates used in mutant, adaptation and de-adaptation studies with triclosan

Isolate	Type	GIC <sup>a</sup>	Original MIC (F/B/V/S/M) <sup>b</sup>	Mutation rate <sup>c</sup>	LDG <sup>d</sup>	MIC ( $\mu\text{g/ml}$ ) to triclosan			
						Parent Isolate	Mutant isolate	After adaptation	After de-adaptation
7266444	<i>S. Enteritidis</i>	No	8/60/60/30/125	$4.9 \times 10^{-10}$	20/20 <sup>e</sup>	0.25/0.25/0.125 <sup>f</sup>	32.2/32.2 <sup>e</sup>	>128.8/>128.8 <sup>f</sup>	>128.8/>128.8 <sup>f</sup>
9675922	<i>S. Infantis</i>	No	8/60/60/30/125	$1.4 \times 10^{-10}$	20/20	0.25/0.25/0.125	32.2/16.1	>128.8/>128.8	>128.8/>128.8
9578243	<i>S. Tennessee</i>	No	8/60/60/30/125	$5.2 \times 10^{-10}$	1/8	0.50/0.25/0.25	32.2/32.2	0.125/0.50	0.50/>128.8
S 4880 98	<i>S. Typh.</i> , DT104	No	8/125/125/60/250	$1.3 \times 10^{-10}$	20/20	0.50/0.25/0.25	32.2/32.2	>128.8/>128.8	>128.8/32.2
9572762	<i>S. Tennessee</i>	No	30/125/250/60/250	$3.7 \times 10^{-9}$	20/20	0.50/0.25/0.25	32.2/32.2	32.2/>128.8	>128.8/>128.8
S 8827 97	<i>S. Senftenberg</i>	No	30/125/250/60/500	$2.0 \times 10^{-9}$	8/20	0.50/0.25/0.25	>128.8/32.2	2.0/>128.8	2.0/>128.8
9577210	<i>S. Tennessee</i>	Yes	30/250/250/60/250	$2.0 \times 10^{-8}$	20/20	2.0/1.0/0.50	<b>64.4/128.8</b>	>128.8/>128.8	>128.8/>128.8

<sup>a</sup> Growth in cyclohexane (parent isolates).<sup>b</sup> Units, cf. Table 3. F, formaldehyde; B, Bio Komplet Plus; V, Virkon S; S, Farm Fluid Super; M, FAM 30.<sup>c</sup> Proportion of cells growing on agar containing 4.0  $\mu\text{g/ml}$  triclosan (cf. text).<sup>d</sup> Last day of growth during adaptation to triclosan.<sup>e</sup> Replicate 1/replicate 2 (applies to the entire column).<sup>f</sup> MICs to triclosan in triplicate (applies to the entire column). MICs for mutant, adapted and de-adapted isolates: Bold numbers show isolates that grew in cyclohexane in both duplicate tests (except >128.8 for isolate S 4880 98 after de-adaptation which grew in only one duplicate test); numbers not in bold show isolates that did not grow in cyclohexane in any of the duplicate tests.

both cyclohexane and *n*-hexane, *E. coli* AG100 grew in only *n*-hexane, whereas *E. coli* NCTC 10418 did not grow in any of these (data not shown).

All 60 isolates grew in *n*-hexane, but only one isolate grew in cyclohexane (data not shown).

### 3.6. Growth in cyclohexane (after triclosan studies)

Among the seven isolates used for the triclosan studies, only the 1/60 isolate growing in cyclohexane was cyclohexane resistant after selection on agar plates with 4.0  $\mu\text{g/ml}$  triclosan (Table 8). However, most of the isolates that grew until day 20 in the triclosan adaptation studies became cyclohexane resistant, and this was maintained after de-adaptation (Table 8). All cyclohexane resistant isolates also grew in *n*-hexane, whereas a few of the cyclohexane sensitive isolates did not grow in *n*-hexane either (data not shown).

## 4. Discussion

Generally, disinfectants act on microorganisms at multiple target sites, but exact mechanisms or key event(s) that cause growth retardation or death of the cell are not well known (Maillard, 2002). In general, resistance to disinfectants is defined as a strain being either insusceptible to a concentration of the

disinfectant used in practice or is not inhibited by a concentration that inhibits the majority of strains of that organism (Russell, 1999). Resistance to disinfectants is considered to be mainly intrinsic, and in Gram-negative bacteria the outer membrane with its LPS-layer is considered to be the main barrier against outer detrimental conditions such as heat and chemicals (Vaara, 1992; Russell, 1999; Maillard, 2002). Many studies have been conducted on barrier mechanisms related to the LPS-layer, but the role of disinfectant resistance in an epidemiological context has not been well elucidated. MIC studies test a bacteriostatic, and not a bactericidal, effect, and disinfectant in-use concentrations are in most cases at least 1000 times the MICs (which are normally increased only by a factor 2–8 in resistant strains) (Russell, 1999; Russell and McDonnell, 2000; Maillard, 2002; Gradel et al., 2004). However, the main purpose of this study was a preliminary screening to detect putative differences between *Salmonella* serotypes and isolates and relate these to persistence and use of disinfectants, for which comprehensive data exist for Danish broiler flocks (AM database, Danish Poultry Council)(Angen et al., 1996; Skov et al., 1999), thus putting emphasis on comparisons, and not on sheer values.

Most epidemiological studies on disinfectant resistance have been from either hospital wards or

the food-processing sector. In MIC studies involving *E. coli*, these have generally been equally sensitive to a number of disinfectants and antiseptics (Stickler and Thomas, 1980; Hammond et al., 1987; Holah et al., 2002; Sidhu et al., 2002). In the former study, higher MICs were mainly found among *Proteus*, *Providencia* and *Pseudomonas* (Stickler and Thomas, 1980). Langsrud and Sundheim (1999) found higher resistance to quaternary ammonium compounds (QACs) in *Pseudomonas* spp. isolated from a poultry processing plant where QACs were used than from a plant using chlorine. Willingham et al. (1996) tested a panel of bacterial isolates from chicken hatcheries in suspension tests with glutaraldehyde, phenol or QAC. The highest resistance level was seen for glutaraldehyde, and this was related to the common use of this type of disinfectant in the hatcheries. A Norwegian study tested *Salmonella* isolates persisting in fish feed factories against nine disinfectants used in these, but did not detect higher resistance than among *Salmonella* from other sources (Møretrø et al., 2003). Recently, *S. Enteritidis* isolates persisting in egg layer houses were found to be equally sensitive to a number of disinfectants as laboratory strains of the same serotype (Davison et al., 2003). Thus, in general little resistance has been detected in field isolates, and it is conspicuous that the few studies with higher disinfectant resistance involved *Pseudomonas*, *Proteus* or *Providencia*, i.e., genera known for their high intrinsic resistance (Guerin-Mechin et al., 1999).

In this study, there were generally few variations in MICs to the five disinfectants used commonly in the Danish or English poultry sector, albeit there were strong associations between high or low MICs when most disinfectants were compared pair wise. There were no obvious associations between MICs on one side and tendencies to persist or the use of relevant disinfectants on the other. A few serotypes (*S. Tennessee*, *S. 4.12:b:-* and *S. Senftenberg*) tended to have higher MICs to some disinfectants, but not necessarily the ones they had encountered beforehand. In general, these results conform to the above studies, i.e., resistance could not be linked epidemiologically to persistence or the frequent use of a few types of disinfectants. Thus, the higher MICs of some serotypes seem to be intrinsic. Our study differed from most of the others by focusing on the primary agricultural sector and disinfectants normally considered more potent

(hence normally not allowed in, e.g., food premises or hospitals). As many disinfectants were combination products, and ancillary substances (e.g., methanol in the formaldehyde solution) are detrimental per se, we do not know if the same mechanisms are involved for related disinfectants. Formaldehyde and glutaraldehyde are both aldehydes, but *S. 4.12:b:-* only had higher MICs to Bio Komplet Plus, whereas *E. coli* NCTC 10418 had higher MICs to formaldehyde but lower MICs to Bio Komplet Plus, maybe because BC in the latter disinfectant also influenced the MICs. These and other differences in MICs illustrate the different uptake and resistance mechanisms that may be involved (Maillard, 2002).

Laboratory adaptation and de-adaptation studies have mainly been performed with chlorhexidine and QACs (Russell, 1998). Adaptation to these disinfectants can increase MICs to them, and this resistance was maintained after several passages in broth without chlorhexidine or QAC (Langsrud, 1998; Sidhu, 2001), though this stability has been questioned by others (Russell, 1998). In our study, no increase in MICs was seen after growth in increasing concentrations of the five disinfectants, for which no previously published adaptation and de-adaptation studies exist to our knowledge. These results consolidate the MIC tests in which no clear development of resistance under real-life conditions was seen either.

In recent years, the multiple antibiotic resistance (*mar*) response has received much attention (Levy, 2002; Randall and Woodward, 2002) after Moken et al. (1997) selected *Mar* mutants of *E. coli* with decreased susceptibility to pine oil (i.e., a phenolic compound). Several studies on the involvement of *mar* in increased resistance to unrelated antibiotics and disinfectants have been conducted, mainly with *E. coli* (Levy, 2002), but *Salmonella* has also been tested (Randall et al., 2001; Randall and Woodward, 2001a,b; Liebana et al., 2002). The *mar*-locus has been shown to be involved in resistance to a few disinfectants (pine oil, triclosan, certain oxidative stress agents) as well as the organic solvent cyclohexane (Asako et al., 1997; Randall and Woodward, 2002), but its role for disinfectants more relevant in the agricultural sector has not been elucidated.

In our study, several results indicated that *mar* did not play a significant role: MICs were very similar between *E. coli* AG100 and AG102 (the *mar*-mutant of

AG100). Cyclohexane resistance was only observed in 1/60 isolates selected for their high MICs. Several isolates became resistant to triclosan, including isolates adapted to this biocide, which also grew in cyclohexane. This latter resistance pattern is known as MAR type resistance, indicative of up-regulated efflux, but only genotypic studies can elucidate which genes (e.g., *acrAb*, *marRAB*, or *soxRS*) were up-regulated. However, it was apparent that isolates that developed the MAR phenotype did not show increased resistance to the five disinfectants. This suggests that efflux mediated resistance is not an important mechanism in resistance to the five disinfectants.

In conclusion, the small variations in MICs to disinfectants used commonly in the poultry sector could not be related clearly to persistence of *Salmonella* or the use of disinfectants, adaptations to these disinfectants did not alter MICs, and up-regulated efflux mechanisms did not seem to be involved in their higher MICs.

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## References

- Angen, Ø., Skov, M.N., Chriél, M., Agger, J.F., Bisgaard, M., 1996. A retrospective study on *Salmonella* infection in Danish broiler flocks. *Prev. Vet. Med.* 26, 223–237.
- Anon., 1997. Microsoft Access, Version 97, 1989–1997. Microsoft Corporation.
- Anon., 2001. Annual Report on Zoonoses in Denmark 2000. Ministry of Food, Agriculture, and Fisheries, Denmark.
- Anon., 2002. UNISTAT Statistical Package [5.5.00]. UNISTAT Ltd., London, UK.
- Anon., 2003. Annual Report on Zoonoses in Denmark 2002. Ministry of Food, Agriculture and Fisheries, Denmark.
- Asako, H., Nakajima, H., Kobayashi, K., Kobayashi, M., Aono, R., 1997. Organic solvent tolerance and antibiotic resistance increased by overexpression of *marA* in *Escherichia coli*. *Appl. Environ. Microbiol.* 63, 1428–1433.
- Bisgaard, M., 1992. A voluntary *Salmonella* control programme for the broiler industry, implemented by the Danish Poultry Council. *Int. J. Food Microbiol.* 15, 219–224.
- Christensen, J.P., Brown, D.J., Madsen, M., Olsen, J.E., Bisgaard, M., 1997. Hatchery-borne *Salmonella enterica* serovar Tennessee infection in broilers. *Avian Pathol.* 26, 155–168.
- Davies, R.H., Breslin, M., Corry, J.E.L., Hudson, W., Allen, V.M., 2001. Observations on the distribution and control of *Salmonella* species in two integrated broiler companies. *Vet. Rec.* 149, 227–232.
- Davies, R.H., Wray, C., 1994. An approach to reduction of *Salmonella* infection in broiler chicken flocks through intensive sampling and identification of cross-contamination hazards in commercial hatcheries. *Int. J. Food Microbiol.* 24, 147–160.
- Davies, R.H., Wray, C., 1995. Observations on disinfection regimens used on *Salmonella* Enteritidis infected poultry units. *Poult. Sci.* 74, 638–647.
- Davies, R.H., Wray, C., 1997. Distribution of *Salmonella* contamination in ten animal feedmills. *Vet. Microbiol.* 51, 159–169.
- Davison, S., Benson, C.E., Munro, D.S., Rankin, S.C., Ziegler, A.E., Eckroade, R.J., 2003. The role of disinfectant resistance of *Salmonella enterica* serotype Enteritidis in recurring infections in Pennsylvania egg quality assurance program monitored flocks. *Avian Dis.* 47, 143–148.
- Gradel, K.O., Rattenborg, E., 2003. A questionnaire-based, retrospective field study of persistence of *Salmonella* Enteritidis and *Salmonella* Typhimurium in Danish broiler houses. *Prev. Vet. Med.* 56, 267–284.
- Gradel, K.O., Sayers, A.R., Davies, R.H., 2004. Surface disinfection tests with *Salmonella* and a putative indicator bacterium, mimicking worst-case scenarios in poultry houses. *Poult. Sci.* 83, 1636–1643.
- Guerin-Mechin, L., Dubois-Brissonnet, F., Heyd, B., Leveau, J.Y., 1999. Specific variations of fatty acid composition of *Pseudomonas aeruginosa* ATCC 15442 induced by quaternary ammonium compounds and relation with resistance to bactericidal activity. *J. Appl. Microbiol.* 87, 735–742.
- Hammond, S.A., Morgan, J.R., Russell, A.D., 1987. Comparative susceptibility of hospital isolates of Gram-negative bacteria to antiseptics and disinfectants. *J. Hosp. Infec.* 9, 255–264.
- Holah, J.T., Taylor, J.H., Dawson, D.J., Hall, K.E., 2002. Biocide use in the food industry and the disinfectant resistance of persistent strains of *Listeria monocytogenes* and *Escherichia coli*. *J. Appl. Microbiol. Suppl.* 92, 111S–120S.
- Langsrud, S., 1998. Resistance to disinfectants used in the food processing industry, with emphasis on resistance to benzalkonium chloride. Thesis, Department of Biotechnological Sciences, Agricultural University of Norway.
- Langsrud, S., Sundheim, G., 1999. Factors contributing to the survival of poultry associated *Pseudomonas* spp. exposed to a quaternary ammonium compound. *J. Appl. Microbiol.* 82, 705–712.
- Levy, S.B., 2002. Active efflux, a common mechanism for biocide and antibiotic resistance. *J. Appl. Microbiol. Suppl.* 92, 65S–71S.
- Liebana, E., Clouting, C., Cassar, C.A., Randall, L.P., Walker, R.A., Threlfall, E.J., Clifton-Hadley, F.A., Ridley, A.M., Davies, R.H., 2002. Comparison of *gyrA* mutations, cyclohexane resistance, and the presence of class I integrons in *Salmonella enterica* from

- farm animals in England and Wales. *J. Clin. Microbiol.* 40, 1481–1486.
- Linton, A.H., Hugo, W.B., Russell, A.D., 1987. *Disinfection in Veterinary and Farm Animal Practice*. Blackwell Scientific Publications, London.
- Maillard, J.Y., 2002. Bacterial target sites for biocide action. *J. Appl. Microbiol. Suppl.* 92, 16S–27S.
- Moken, M.C., McMurry, L.M., Levy, S.B., 1997. Selection of multiple-antibiotic-resistant (*mar*) mutants of *Escherichia coli* by using the disinfectant pine oil: roles of the *mar* and *acrAB* loci. *Antimicrob. Agents Chemother.* 41, 2770–2772.
- Møretrø, T., Midtgaard, E.S., Nesse, L.L., Langsrud, S., 2003. Susceptibility of *Salmonella* isolated from fish feed factories to disinfectants and air-drying at surfaces. *Vet. Microbiol.* 94, 207–217.
- Randall, L.P., Cooles, S.W., Sayers, A.R., Woodward, M.J., 2001. Association between cyclohexane resistance in *Salmonella* of different serovars and increased resistance to multiple antibiotics, disinfectants and dyes. *J. Med. Microbiol.* 50, 919–924.
- Randall, L.P., Woodward, M.J., 2001a. Multiple antibiotic resistance (*mar*) locus in *Salmonella enterica* serovar Typhimurium DT104. *Appl. Environ. Microbiol.* 67, 1190–1197.
- Randall, L.P., Woodward, M.J., 2001b. Role of the *mar* locus in virulence of *Salmonella enterica* serovar Typhimurium DT104 in chickens. *J. Med. Microbiol.* 50, 770–779.
- Randall, L.P., Woodward, M.J., 2002. The multiple antibiotic resistance (*mar*) locus and its significance. *Res. Vet. Sci.* 72, 87–93.
- Russell, A.D., 1998. Mechanisms of bacterial resistance to antibiotics and biocides. *Prog. Med. Chem.* 35, 133–197.
- Russell, A.D., 1999. Bacterial resistance to disinfectants: present knowledge and future problems. *J. Hosp. Infect. Suppl.* 43, S57–S68.
- Russell, A.D., McDonnell, G., 2000. Concentration: a major factor in studying biocidal action. *J. Hosp. Infect.* 44, 1–3.
- Sidhu, M.S., 2001. Disinfectant- and antibiotic resistant bacteria from food and clinical environments. Thesis, Department of Chemistry and Biotechnology, Agricultural University of Norway.
- Sidhu, M.S., Sørum, H., Holck, A., 2002. Resistance to quaternary ammonium compounds in food related bacteria. *Microb. Drug Resist.* 8, 393–399.
- Skov, M.N., Angen, Ø., Chriél, M., Olsen, J.E., Bisgaard, M., 1999. Risk factors associated with *Salmonella enterica* serovar Typhimurium infection in Danish broiler flocks. *Poult. Sci.* 78, 848–854.
- Stickler, D.J., Thomas, B., 1980. Antiseptic and antibiotic resistance in Gram-negative bacteria causing urinary tract infection. *J. Clin. Pathol.* 33, 288–296.
- Strauch, D., Böhm, R., 2002. *Reinigung und Desinfektion in der Nutztierhaltung und Veredelungswirtschaft*. Enke Verlag, Stuttgart, Germany.
- Vaara, M., 1992. Agents that increase the permeability of the outer membrane. *Microbiol. Rev.* 56, 395–411.
- White, D.G., Goldman, J.D., Demple, B., Levy, S.B., 1997. Role of the *acrAB* locus in organic solvent tolerance mediated by expression of *marA*, *soxS*, or *robA* in *Escherichia coli*. *J. Bacteriol.* 179, 6122–6126.
- Willingham, E.M., Sander, J.E., Thayer, S.G., Wilson, J.L., 1996. Investigation of bacterial resistance to hatchery disinfectants. *Avian Dis.* 40, 510–515.