MINIMUM INHIBITORY CONCENTRATIONS OF AND ADAPTATION TO FIVE DISINFECTANTS COMMONLY USED AGAINST *SALMONELLA* IN THE POULTRY INDUSTRY

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Abstract

MIC-tests in replicate were performed on 286 *Salmonella* isolates (269 from Danish poultry, including 256 from broiler houses, and 17 from England, mainly from poultry) against five disinfectants used in the poultry industry (glutaraldehyde/benzalkonium chloride compound, formaldehyde, oxidising compound, tar oil phenol, iodophor). Generally, the small variations in MIC could not be associated with previous use of disinfectants, persistence or serotypes. Adaptation studies involving the five disinfectants did not alter the MICs beyond the normal biological variation, i.e. within one doubling dilution.

INTRODUCTION

In Denmark, samples for *Salmonella* examination have been submitted from all broiler flocks since 1992 (Bisgaard 1992; Anonymous 2002) and data recordings from each flock, including the use of disinfectants, were made for the same period (Skov *et al.* 1999). Some *Salmonella* serotypes have persisted in Danish broiler houses for years, while others have been eliminated after one or a few crops. Little research has been done on resistance to disinfectants and its relation to persistence in animal houses.

The aims of this study were:

- To find minimum inhibitory concentrations (MICs) of five disinfectants for "non-persistent" and "persistent" *Salmonella* serotypes commonly isolated from Danish broiler houses.
- To find MICs of the five disinfectants for other *Salmonella* serotypes mainly isolated from poultry enterprises.
- To perform adaptation and de-adaptation studies with the five disinfectants for selected strains having high or low MICs to see if disinfectant resistance was developed and maintained.

MATERIALS AND METHODS

Strains (Table 1)*:*

Danish *Salmonella* strains were stored in Standard-Keimzahlager (Merck 1.01621), while English strains were stored on Dorset's egg slopes, all at room temperature. Three strains of *Escherichia coli* (NCTC 10418, AG100, AG102) were used as controls in all MIC-tests.

Table 1. *Sources of bacterial isolates*

Country	Type	Number of isolates	Source and description
Denmark	S.[1] Enteritidis	34	Danish broiler houses, "non-persistent type"
Denmark	S. Typhimurium	39	Danish broiler houses, "non-persistent type"
Denmark	S. Tennessee	24	Danish broiler houses, "non-persistent type"
Denmark	S. 4.12:b:-	81	Danish broiler houses, "persistent type"
Denmark	S. Infantis	61	Danish broiler houses, "persistent type"
Denmark	S. Indiana	17	Danish broiler houses, "persistent type"
Denmark	S. Senftenberg	13	Poultry sector
UK	S. Choleraesuis NCTC 10653	1	Strain used in English disinfection tests
UK	S. Typhimurium, DT104	8	Pig and broiler farms
UK	S. 4.12:d:-	4	Feed mill and hatchery
UK	S. Senftenberg	4	Hatchery
UK	*E. coli* NCTC 10418	1	Control strain
UK	*E. coli* AG100	1	Control strain
UK	*E. coli* AG102	1	Control strain, *mar*[2] mutant of *E. coli* AG100

[1] *Salmonella.*

[2] *Multiple antibiotic resistance regulon which upregulates the AcrAB efflux pump (Levy 2002).*

All English isolates, except S. Choleraesuis NCTC 10653, were from samples taken both before and after disinfection. Because the English S. Senftenberg isolates had high MICs, Danish isolates were included to see if this applied generally to this serotype.

Epidemiology of Salmonella from Danish broiler houses (Table 2).

The source of S. Enteritidis, S. Typhimurium and S. Tennessee infections in Danish poultry was usually day-old chicks (Christensen *et al.* 1997; Gradel and Rattenborg 2002); in most cases these were eliminated in one or two crops, i.e. "non-persistent" types. The sources of S. Infantis, S. 4.12:b:- and S. Indiana were more difficult to trace (Gradel and Rattenborg 2002), although feed has been suspected (Angen *et al.* 1996). These types have persisted in several crops in quite a few Danish broiler houses and farms, i.e. "persistent" types. However, in this study "non-persistent" and "persistent" types that were found in more/less than two crops, respectively, were also selected. From most of the houses shown in Table 2, two or more isolates were selected, representing both the beginning and the end of a persistence period.

Table 2. *Persistence of Salmonella types from broiler houses used in this study, from 3/1/92 to 2/10/01*

Salmonella type	Number of crops with the same *Salmonella* type								
	1	2	3	4	5	6-10	11-20	21-30	> 30
Enteritidis		5[1]	6	4		2			
Typhimurium	2	7	4	3	1	2	2		
Tennessee		4	4	1	1	2			
4.12:b:-	1	3	2	2	4	6	4	5	1
Infantis		9	2	3	3	6	4		
Indiana	1	1	2	1	1	3			

[1] *numbers of broiler houses*

Disinfectants:

There were 4,629 *Salmonella* positive Danish broiler flocks in the period 3/1/92 to 2/10/01. Among these, a glutaraldehyde/benzalkonium chloride compound, formaldehyde and an oxidising compound were used most commonly for disinfection of the broiler houses (38.8, 32.4 and 14.9%, respectively). Phenols and iodophors were used very rarely in Danish poultry houses. In the UK, phenols were used commonly in poultry houses while iodophors were used mainly for foot dips and in hatcheries (R. Davies, pers. comm.). Therefore, a glutaraldehyde (23%) and benzalkonium chloride (5%) compound (GB), formalin (24.5% formaldehyde) (F), an oxidising compound (blend of peroxygen compounds) (O), a phenol compound (30-45% high boiling tar acids) (P) and an iodophor (I) were chosen for this study. All disinfectants, except F, were branded products. O was a powder, while the others were liquids.

MIC-tests:

MICs were performed as previously described (Randall *et al*. 2001). For all *Salmonella* isolates, the tests were performed at least in duplicate on different days. The *E. coli* control strains were included in each batch to check for deviations between batches.

Adaptation tests:

Six isolates, three with high and three with low MICs, were used for 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 at 37 °C. A 0.1 ml inoculum was added to 3.0 ml LB with a disinfectant concentration half the lowest recorded MIC which was then incubated overnight at 37 °C. Each consecutive day the disinfectant concentration in LB was increased by a factor of 1.5 and a 0.1 ml inoculum from the LB broth grown the previous day was passaged to this. Turbidity was registered visually and the culture was streaked on blood agar (BA) 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 supernatant was discarded and the pellet was suspended in physiological saline to McFarland 0.5 for MIC-tests. The isolates were then passaged in 3.0 ml LB broth without disinfectant for six consecutive days, after which the MIC-tests were repeated.

Statistical analysis:

All data were recorded into an Access database (Anonymous 1997). Differences were tested by chi-square or 2-tailed Fisher exact tests (for expected values < 5) and associations by McNemar chi-square tests (Cochran 1950), all using 95% significance limits, and by Cohen's Kappa (Sackett 1992).

RESULTS AND DISCUSSION

MIC-tests:

Generally, for all five disinfectants there were few variations in MICs between serotypes, sources and countries (Table 3). The disinfectants F, O and I had significantly higher MICs to *S.* Tennessee (i.e. a "non-persistent" serotype), disinfectant F had significantly higher MICs to *S.* Senftenberg, and disinfectant I (which was used rarely in Danish poultry houses) had significantly higher MICs to *S.* 4.12:b:- (all $p<0.01$). The five disinfectants had high MICs to the four English *S.* Senftenberg; however, MICs were only significantly higher for the disinfectants F ($p=0.0007$) and O ($p=0.0040$).

Table 3. *MICs for Salmonella isolates*

Country	*Salmonella* type	Disinfectants (see text for designations) and MICs									
		F		O		GB		P		I	
		Low[1]	High[1]	Low	High	Low	High	Low	High	Low	High
Denmark	Enteritidis	34[2]	0	28	6	14	20	13	21	13	21
Denmark	Typhimurium	39	0	36	3	20	19	19	20	22	17
Denmark	Tennessee	9	15	8	16	6	18	4	20	2	22
Denmark	4.12:b:-	66	15	59	22	12	69	17	64	14	67
Denmark	Infantis	61	0	43	18	18	43	29	32	17	44
Denmark	Indiana	16	1	16	1	11	6	16	1	14	3
Denmark	Senftenberg	0	13	10	3	5	8	1	12	10	3
UK	Choleraesuis	1	0	1	0	0	1	1	0	1	0
UK	Typh., DT104	8	0	8	0	0	8	0	8	2	6
UK	4.12:d:-	4	0	4	0	0	4	0	4	0	4
UK	Senftenberg	0	4	0	4	0	4	0	4	0	4

[1] All MICs are ml/100 ml except for disinfectant O (g/100 ml). MICs are grouped in this Table (F: Low=0.004 and 0.008, High=0.015 and 0.030; O: Low=0.060 and 0.125, High=0.250; GB: Low=0.060, High=0.125 and 0.250; P: Low=0.015 and 0.030, High=0.060 and 0.125; I: Low=0.060 and 0.125, High=0.250 and 0.50).
[2] numbers of isolates.

For the three disinfectants F, O and I which showed significant differences in MICs for some serotypes, cross-tabulations were made in order to deduce putative associations between MICs. No strong associations were seen (all McNemar $p>0.05$ and Cohen's Kappa in the range 0.02-0.29 - data not shown).

A total of 67 and 21 broiler houses were represented with two or more isolates of the same serotype, respectively. For each house, increase, decrease or no change in MICs was registered. There were no significant changes in MICs between isolates within the same houses, either generally ($p=0.30$) or for any of the serotypes (data not shown), i.e. apparently no disinfectant resistance developed during the period where *Salmonella* prevailed in the broiler house.

In addition, no associations were seen between MICs and the use of the three "Danish" disinfectants (GB, F or O) recorded in the database in the preceding download period (data not shown).

Results for the three *E. coli* control strains illustrate the variability between test days and strains. The variability within strains was within one doubling dilution, i.e. normal biological variance. There were no differences in MICs between AG100 and AG102, suggesting that the *mar*

response (Levy 2002) was not involved in resistance against the disinfectants studied. NCTC 10418 had higher MICs than the other two strains for disinfectants O and I, while it was more sensitive for disinfectant GB, indicating that uptake and resistance mechanisms are different for different disinfectant types (Maillard 2002).

Adaptation studies:

Although growth was detected in concentrations up to ca. 13 times the original MIC, this was not reflected in equivalent MICs after adaptation (data not shown). Generally, decreased MICs after adaptation were seen for the three isolates having original high MICs, while increases were seen for the three isolates with original low MICs. Nearly all changes were within one doubling dilution, both after adaptation and de-adaptation.

In conclusion, the small variations in MIC observed could not be related clearly to *Salmonella* serotype, persistence or the use of specific disinfectants. Few other disinfectant resistance studies have dealt with bacterial field isolates and disinfectants commonly found in animal houses. Other studies have dealt with bacteria from hospital wards (Stickler and Thomas 1980; Hammond *et al.* 1987) or the food industry (Holah *et al.* 2002; Nesse *et al.* 2002). The results from those studies confirm the ones reported here, i.e. the little disinfectant resistance observed could not be associated clearly with bacterial persistence. Others have adapted bacteria to mainly chlorhexidine or quaternary ammonium compounds (QACs) in the laboratory (Langsrud 1998; Sidhu 2001) but the stability of this resistance has been questioned (Russell 1998). Here, only few MIC changes were reported after adaptation studies with five disinfectants normally considered more detrimental for bacteria than QACs, but more research is needed on this topic.

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