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Effect of sub-lethal concentrations of biocides on the susceptibility to antibiotics of multi-drug resistant *Salmonella enterica* strains

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ABSTRACT

Ten multi-drug resistant *Salmonella enterica* isolates belonging to ten different serotypes were exposed to increasing sub-inhibitory concentrations of three biocides widely used in food industry facilities (trisodium phosphate, sodium nitrite, or sodium hypochlorite). Cultures were tested, before and after exposure to biocides, against 31 antibiotics of clinical significance by means of a standard disk-diffusion technique (CLSI). Exposed cultures displayed reduced susceptibility to a range of antibiotics, as compared with not exposed cultures. The impact of biocide exposure on reduced susceptibility to antibiotics was dependent on the *Salmonella* strain and the antibiotic family tested, susceptibility to aminoglycosides and cephalosporins being the most strongly affected. Results in the present study suggest that extensive use of biocides at sub-lethal concentrations could contribute to the emergence of antibiotic resistance in multi-drug resistant *Salmonella enterica* strains and therefore represent a public health risk. The intra-specific differences observed in antibiotic susceptibility underline the need to screen a wide range of strains.

1. Introduction

Non-typhoidal *Salmonella* caused over 95,500 confirmed cases of enteritis in the European Union in 2011 (the notification rate was 20.7 cases per 100,000 population), resulting in significant morbidity and 56 deaths (EFSA, 2013). While most cases of human salmonellosis are self-limiting, requiring only bed rest and re-hydration, antibiotic therapy may be necessary for severe cases, extra-intestinal disease or immune-compromised patients (Álvarez-Fernández, Alonso-Calleja, García-Fernández, & Capita, 2012). In such a scenario, *Salmonella* strains that are resistant to commonly used antibiotics are especially threatening because they may compromise the effective treatment of human illness.

Biocides are used extensively in the food industry as food additives, decontaminants or environmental disinfectants to control harmful micro-organisms (Capita & Alonso-Calleja, 2013). Sub-inhibitory concentrations of biocides could occur as consequence of improper use (erroneous concentration or inadequate distribution), inappropriate storage of the formulations, excessive amounts of organic matter (known to inactivate several biocides) or use of compounds at sub-lethal concentrations, leading to a "hurdle effect" (Alonso-Hernando, Capita, Prieto, & Alonso-Calleja, 2009). Recent scientific evidence suggests that the selective pressure exerted by the use of biocides, including compounds widely used in food production facilities, could select for resistance to antibiotics, e.g. cross-resistance, co-resistance, selection for clones that are resistant to both biocides and antibiotics, or activation of the SOS response (Capita & Alonso-Calleja, 2013; Randall et al., 2007). In view of the large and increasing use of biocides (generally available figures show that the consumption of biocides in the European Union increased by 4%–5% per year in the last decade, with a market of €10 billion to €11 billion in 2006; SCENIHR, 2009), the risk of biocide use leading to the selection and spread of antibiotic resistant bacteria is of increasing concern. However, scientific evidence is therefore insufficient to assess the risk of biocides correctly (SCENIHR, 2009). Therefore, the aim of this research was to determine the effect of exposure to increasing sub-inhibitory concentrations of three biocides commonly used in the Food Industry (trisodium phosphate, sodium nitrite and sodium hypochlorite) on the susceptibility of multi-drug resistant *Salmonella enterica* isolates from poultry to a range of antibiotics of clinical significance.

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2. Materials and methods

2.1. Strains

Ten multi-drug resistant *S. enterica* strains (with resistance to between 6 and 15 antibiotics) previously isolated from poultry were used in this study: *Salmonella* Agona (SA1), *Salmonella* Anatum (SA2), *Salmonella* Enteritidis (SE), *Salmonella* Hadar (SH), *Salmonella* Infantis (SI), *Salmonella* Kentucky (SK), *Salmonella* Thompson (ST1), *Salmonella* Typhimurium (ST2), *Salmonella* 1, 4, (5), 12: i- (S1), and *Salmonella* Virchow (SV). Strains were maintained in tryptone soya broth (TSB, Oxoid Ltd., Hampshire, England) with 20% (v/v) glycerol at −30 °C. Working cultures were kept at 4 ± 1 °C on plates of tryptone soya agar (TSA, Oxoid).

2.2. Biocides

Three compounds were tested: trisodium phosphate (TSP, Merck, Darmstadt, Germany), sodium nitrite (SNI, Sigma–Aldrich, Steinheim, Germany) and sodium hypochlorite (SHY, Sigma–Aldrich). These compounds are used in the Food Industry as food preservatives (TSP and SNI), decontaminants (TSP) or disinfectants (SHY). Solutions were aseptically prepared before each experiment in sterile distilled water.

2.3. Determination of the Minimum Inhibitory Concentrations (MICs)

The Minimum Inhibition Concentration (MIC) values were established using a microdilution broth method following the Clinical and Laboratory Standards Institute guidelines (CLSI, 2008). Five colonies of each organism were taken from TSA plates, inoculated into 10 mL of Mueller-Hinton (MH) broth (Oxoid) and incubated at 37 °C. After 24 h of incubation these bacterial cultures contained approximately 10^8 cfu/mL. For the experiment, 100-well polystyrene micro-well plates (Oy Growth Curves Ab Ltd., Helsinki, Finland) were used. Wells were filled with 20 μL of the solution of biocides (a range of concentrations was used) and 180 μL of appropriate dilutions (in MH broth) of inocula in order to give a final concentration in the well of 5×10^5 cfu/mL. The inoculum concentration was confirmed by plating. Both positive (200 μL of inoculum at 5×10^5 cfu/mL) and negative (180 μL of MH broth + 20 μL of chemical compound) controls were included in each experiment. Initial optical density at 420–580 nm ($OD_{420–580}$) values ranged from 0.090 to 0.100. No significant differences were observed between bacterial strains, types of biocides, or controls.

The microwell plates were incubated at 37 °C in a Bioscreen C MBR (Oy Growth Curves Ab) and the MIC was established as the lowest biocide concentration necessary to prevent growth after 48 h of incubation. According to previous findings, an $OD_{420–580}$ of 0.200 was considered the cut-off for bacterial growth (Díez-García, Capita, & Alonso-Calleja, 2012). Experiments were replicated five times on separate days.

2.4. Exposure to increasing sub-inhibitory concentrations of biocides

The test was performed in the same manner as described for determining MIC. The biocides starting concentration was MIC/2. When growth was observed, 20 μL of the suspension were aseptically transferred to the next well, which contained 160 μL of MH broth and 20 μL of biocide solution. After the transfer, each well contained a biocide concentration 1.5 times stronger than the previous well. This procedure was continued until no growth was observed after 72 h of incubation at 37 °C. The suspension in the last well with recorded growth was streaked on TSA plates with biocide (one-half of the maximum concentration of biocide that supported microbial growth was added to TSA) and stored (4 ± 1 °C) after incubation at 37 °C for 48 h.

2.5. Antibiotic susceptibility testing

Isolates were screened for susceptibility, before and after exposure to the chemicals, to a panel of 31 antibiotics on Mueller-Hinton agar (Oxoid) by a disk diffusion method described by the National Committee for Clinical Laboratory Standards (CLSI, 2008). The following disks (Oxoid) were used: spectinomycin (SH, 100 μg), amikacin (AK; 30 μg), gentamicin (CN; 10 μg), kanamycin (K, 30 μg), streptomycin (S; 10 μg), tobramycin (TOB, 10 μg), rifampicin (RD, 5 μg), ampicillin (AMP, 10 μg), penicillin G (P, 10 units), ticarcillin (TIC, 75 μg), amoxicillin/clavulanic acid (AMC, 30 μg), ampicillin/sulbactam (SAM, 20 μg), piperacillin/tazobactam (TZP, 110 μg), cephalothin (KF; 30 μg), cefazolin (KZ, 30 μg), cefoxitin (FOX, 30 μg), cefotaxime (CTX, 30 μg), ceftazidime (CAZ, 30 μg), cefepime (FEP, 30 μg), imipenem (IPM, 10 μg), aztreonam (ATM, 30 μg), sulphonamides (S3, 300 μg), trimethoprim/sulphamethoxazole (SXT; 25 μg), teicoplanin (TEC, 30 μg), chloramphenicol (C; 30 μg), nalidixic acid (NA; 30 μg), ciprofloxacin (CIP; 5 μg), enrofloxacin (ENR; 5 μg), tetracycline (TE; 30 μg), phosphomycin (FOS, 50 μg), and nitrofurantoin (F, 300 μg). The inhibition zones were measured and scored as sensitive, intermediate susceptibility and resistant according to the CLSI (2008) guidelines. To control the precision and accuracy of the results obtained with the disk diffusion test procedure cultures of two quality control strains (*Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213) with known antimicrobial resistance patterns were used. A total of 1240 tests (considering all combinations of strains, biocides and antibiotics) were performed. Repeatability was assessed by testing a fourth of tests (including combinations for all strains, biocides and antibiotics) twice on separate days with a 3-month interval.

2.6. Statistical analysis

MIC values were evaluated using analysis of variance techniques. Mean separations were obtained using Duncan's multiple range test. The percentages of cultures with increased resistance to antibiotics after exposure to biocides were compared by means of the Fisher's exact and Chi-square tests. Significance was determined at the $P < 0.05$ level. Data processing was carried out using the Statistica® 6.0 software package (Statsoft Ltd., Chicago, Illinois, USA).

Table 1
Minimum inhibitory concentrations (MICs) of three biocides (mg/mL) obtained for 10 multi-drug resistant *Salmonella enterica* strains.

Salmonella strain	Biocide		
	TSP	SNI	SHY
Salmonella Agona	12.60 ± 0.24ab	12.04 ± 0.09ab	0.43 ± 0.04a
Salmonella Anatum	12.56 ± 0.09ab	12.20 ± 0.35bcd	0.43 ± 0.03a
Salmonella Enteritidis	12.74 ± 0.31a	12.32 ± 0.30bd	0.43 ± 0.04a
Salmonella Hadar	12.34 ± 0.09b	11.88 ± 0.11ac	0.44 ± 0.05a
Salmonella Infantis	12.36 ± 0.09b	11.96 ± 0.22ac	0.43 ± 0.03a
Salmonella Kentucky	12.64 ± 0.33ab	11.88 ± 0.11ac	0.43 ± 0.03a
Salmonella Thompson	12.44 ± 0.09ab	12.44 ± 0.41d	0.44 ± 0.04a
Salmonella Typhimurium	12.48 ± 0.11ab	11.84 ± 0.09a	0.42 ± 0.05a
Salmonella 1, 4, (5), 12: i-	12.48 ± 0.41ab	11.84 ± 0.22a	0.44 ± 0.04a
Salmonella Virchow	12.52 ± 0.27ab	11.96 ± 0.17ac	0.39 ± 0.04a

TSP, Trisodium phosphate; SNI, sodium nitrite; SHY, sodium hypochlorite. Average MICs in the same column with no letters in common are significantly different ($P < 0.05$).