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Comparison of antibiotic resistance patterns in *Listeria monocytogenes* and *Salmonella enterica* strains pre-exposed and exposed to poultry decontaminants

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ABSTRACT

Recent opinions expressed by European Union Scientific Committees suggest there is a potential for poultry decontaminants to increase resistance to antibiotics. At this moment there is no scientific information available to estimate this risk accurately. Four strains (*Listeria monocytogenes* serovar 1/2a, *Listeria monocytogenes* serovar 4b, *Salmonella enterica* serotype Typhimurium and *Salmonella enterica* serotype Enteritidis) were repeatedly exposed to increasing sub-inhibitory concentrations of decontaminants (trisodium phosphate, acidified sodium chlorite, citric acid, chlorine dioxide or peroxyacetic acid) and tested against 15 antibiotics by means of a standard disc-diffusion technique (NCCLS). The antibiotic resistance patterns of strains were compared before and after exposure to decontaminants. Intra-specific differences in antibiotic resistance patterns were found among strains. Increases in resistance to various antibiotics were observed in *L. monocytogenes* and *S. enterica* strains after exposure to chemicals (especially ASC). These results raise concerns over the application of certain poultry decontaminants, since they could contribute to the development of microbial resistance mechanisms. However, these are preliminary results derived from laboratory-based experiments. Additional studies under practical field conditions would be needed to substantiate these findings.

1. Introduction

Red meat and poultry products are frequently contaminated with pathogenic microorganisms. During 2006, these foodstuffs were responsible in the European Union for 25% of reported food-borne outbreaks for which the vehicle involved was known (EFSA, 2007). The prevalence and levels of pathogens on poultry carcasses may be controlled by applying an integrated control strategy over the whole food chain. Provided this strategy is followed, decontamination treatments can constitute a useful element in further reducing the presence of pathogens. Article 3(2) of Regulation (EC) No. 853/2004 of the European Parliament and of the Council laying down specific hygiene rules for foods of animal origin, provides a legal basis to permit "the use of a substance other than potable water" to remove surface contamination from products of animal origin, hitherto prohibited for years in Europe (OJEC, 2004). Permission for such a use must be preceded by a demonstration that the techniques are safe, taking into account the potential pathogenic microflora involved.

In 2008, various European Scientific Committees examined the possible development of antibiotic resistance linked to four substances used to decontaminate poultry carcasses (trisodium phosphate, acidified sodium chlorite, chlorine dioxide and peroxyacids), concluding that there is no specific research on the risk of these treatments triggering resistance to therapeutic antimicrobials (EFSA, 2008a; SCHER/SCENIHR, 2008). In the light of previous scientific opinions, in June 16, 2008, the European Parliament adopted a non-binding resolution against the antimicrobial treatment of poultry carcasses. The precautionary principle is among the causes for this resolution. In December 18, 2008, the European Union Agriculture and Fisheries Council rejected a proposal for a Regulation on the use of antimicrobial substances to remove surface contamination from poultry carcasses.

Antimicrobial-resistant pathogenic bacteria are biological hazards associated with increased human morbidity and mortality rates. Recent scientific evidence suggests that over the last decade, antibiotic resistance through various mechanisms has increased worldwide, posing a serious concern for Public Health (ECDC, 2007; WHO, 2007; EFSA, 2008b; SCENIHR, 2008). The association between chemical biocides (other than poultry decontaminants) and the emergence of resistance to antibiotics has been previously demonstrated. It has been hypothesized that some biocides and antibiotics may share common behaviours and properties in their respective activities and in the resistance mechanisms developed by bacteria (Capita, 2007; SCHER/SCENIHR, 2008; SCENIHR, 2008). Because chemical decontaminants for poultry have not so far been tested in this regard, research on the potential for the occurrence of resistance to therapeutic antimicrobials should be encouraged (EFSA, 2008a).

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In this study it was investigated whether exposure and adaptive tolerance to poultry decontaminants could influence resistance to therapeutic antimicrobials among *Listeria monocytogenes* and *Salmonella enterica* strains.

2. Materials and methods

2.1. Strains

Four strains previously isolated in our laboratory were used in this study: *L. monocytogenes* serovar 1/2a (L1), *L. monocytogenes* serovar 4b (L2), *S. enterica* serotype Typhimurium (S1) and *S. enterica* serotype Enteritidis (S2). With the exception of L2, all strains were derived from poultry carcasses. L2 was obtained from a sheep's spinal cord. Isolates were maintained in tryptic soy broth (TSB, Oxoid) with 20% (v/v) glycerol at −20 °C. Working stock cultures were prepared on tryptic soy agar (TSA, Oxoid) plates incubated at 37 °C. Subcultures were prepared by transferring an isolated colony from a plate into a test tube containing 10 ml of sterile TSB (Oxoid).

2.2. Exposure to poultry decontaminants

Five chemical decontaminants were included in the tests: trisodium phosphate (TSP, Merck, Darmstadt, Germany), sodium chlorite (Fluka, Madrid, Spain) acidified to pH 2.7 by adding citric acid (Panreac, Barcelona, Spain) (acidified sodium chlorite, ASC), citric acid (CA), chlorine dioxide (CD) (Tecsa® Clor, Productos Técnicos Protecsa, S.A., Chile) and peroxyacetic acid (PA) (Inspexx 100, Ecolab, St. Paul, USA). All solutions were aseptically prepared in sterilised distilled water before each experiment. The minimum inhibitory concentration (MIC) values of chemicals for all four strains were established using a micro-dilution broth method following the NCCLS Standards.

Strains were repeatedly passed through media containing increasing concentrations of a given chemical (Alonso-Hernando, Capita, Prieto, & Alonso-Calleja, 2009). The initial concentration of decontaminant in the 200 μl microtiter test wells used was MIC/2. When growth was visually observed, 20 μl of the suspension were aseptically transferred to the next well, which contained 160 μl of Mueller–Hinton broth (MH) and 20 μl of decontaminant solution. After the transfer, each well contained a decontaminant concentration 1.5 times stronger than the previous well. This procedure was continued until no growth was observed after 3 days of incubation at 37 °C. The suspension in the last well with recorded visible growth was centrifuged at 6600*g* for 2 min, and the pellet was washed with phosphate-buffered saline to remove the compound. The pellet was re-suspended in 10 ml of MH broth and incubated at 37 °C for 24 h. Cultures were streaked on TSA plates and stored at 3 ± 1 °C, after incubation at 37 °C for 48 h. The MIC of each chemical decontaminant was established for strains exposed to that compound. Table 1 shows the MIC increases (number-fold), relative to unexposed strains, observed after the exposure experiment.

2.3. Antimicrobial susceptibility testing

Isolates were screened for susceptibility (before and immediately after exposure to decontaminants) to a panel of 15 antibiotics on Mueller–Hinton agar (Oxoid) by a disc-diffusion method described by the National Committee for Clinical and Laboratory Standards (NCCLS, 2000; CLSI, 2002). The following discs (Oxoid) were used: streptomycin (STR; 10 μg), gentamicin (GEN; 10 μg), neomycin (N; 10 μg), rifampicin (RA; 5 μg), ampicillin (AMP; 10 μg), amoxicillin-clavulanic acid (AMC; 30 μg), cephalotin (CF; 30 μg), sulphamethoxazole-trimethoprim (SXT; 25 μg), erythromycin (E; 15 μg), chloramphenicol (C; 30 μg), nalidixic acid (NA; 30 μg), enrofloxacin (EF; 5 μg), ciprofloxacin (CP; 5 μg), tetracycline (TE; 30 μg) and furazolidone (FZ; 50 μg). The inhibition zones were measured and scored as sensitive, intermediate susceptibility and resistant according to the CLSI (2002) guidelines. For STR, neomycin and furazolidone, the criteria of the *Comité de l'Antibiogramme de la Societé Française de Microbiologie* (Sanders, 2006) were used. Cultures of *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 with known antimicrobial resistance patterns were used as reference strains for antibiotic disc control.

Table 1
Increases (number-fold) in MIC, as compared to unexposed strains, observed after exposure to progressively increasing concentrations of decontaminants.

Exposure	Strain			
	L1[a]	L2[b]	S1[c]	S2[d]
TSP[e]	1.12	1.15	1.18	1.18
ASC[f]	2.71	1.88	2.14	2.59
CA[g]	1.31	1.00	1.00	0.90
CD[h]	1.48	0.98	1.13	1.17
PA[i]	1.00	1.10	1.14	1.00

[a] L1, *Listeria monocytogenes* serovar 1/2a (poultry isolate).
[b] L2, *Listeria monocytogenes* serovar 4b (ewe's spinal cord isolate).
[c] S1, *Salmonella enterica* serotype Typhimurium (poultry isolate).
[d] S2, *Salmonella enterica* serotype Enteritidis (poultry isolate).
[e] TSP, trisodium phosphate.
[f] ASC, acidified sodium chlorite.
[g] CA, citric acid.
[h] CD, chlorine dioxide.
[i] PA, peroxyacetic acid.

3. Results and discussion

Considerable controversy surrounds the use of biocides and the possibility that their use to sub-inhibitory concentrations may alter resistance to antibiotics. Sub-inhibitory concentrations could occur when chemicals are used at the lowest authorized concentrations (e.g. ASC), or because of inadequate distribution or dosage of disinfectants, or excessive amounts of organic matter, known to inactivate disinfectants, in the dipping tank (Alonso-Hernando et al., 2009).

In this study, the antibiotic resistance patterns of decontaminant-exposed cultures were compared, for the first time, with those of unexposed cultures by use of a standard disc-diffusion technique.

After exposure to increasing sub-inhibitory concentrations of decontaminants, *L. monocytogenes* and *S. enterica* strains exhibited an increase in their resistance to various antibiotics when compared to unexposed isolates (Table 2). In some cases, the resistance expressed moved strains from the category of "sensitive" to that of "resistant" according to the guidelines used. This was particularly the case for streptomycin (strain S1 exposed to ASC, CD and PA; and S2 exposed to ASC), cephalotin (L1 exposed to ASC) and chloramphenicol (L1 and L2 exposed to ASC). In other cases, the level of adaptive resistance was not as pronounced, moving strains from "sensitive" to "intermediately sensitive", or from "intermediately sensitive" to "resistant". For various additional strains and antibiotics, the diameter of inhibition zones in exposed cells decreased with regard to unexposed, but remained well above the limit for resistance. According to Braoudaki and Hilton (2004), even a level of increased resistance below the criteria set by the NCCLS would still be significant, since a modest change in susceptibility may ultimately confer a growth advantage on a strain.

As in the research being reported here, Walsh et al. (2003), also observed that exposure to biocides changed the categorization of

Table 2
Antibiotic resistance patterns of strains tested.

Strain	Antibiotic														
	STR	GEN	N	RA	AMP	AMC	CF	SXT	E	C	NA	EF	CP	TE	FZ
L1[a] non-exposed	I		I								R	I			
L1-exposed to TSP[e]	I		I								R	I			
L1-exposed to ASC[f]	I		I				R			R	R	I	I		
L1-exposed to CA[g]	I		I								R	I			
L1-exposed to CD[h]	R		I								R	I			
L1-exposed to PA[i]	I		I								R	I			I
L2[b] non-exposed											R				
L2-exposed to TSP	I		I								R	I			I
L2-exposed to ASC			I							R	R	I			
L2-exposed to CA											R	I			
L2-exposed to CD											R	I			
L2-exposed to PA	I										R	I	I		
S1[c] non-exposed			R	R			I		R		R	I			
S1-exposed to TSP	I		R	R			I		R		R	I			
S1-exposed to ASC	R		R	R	I		I		R		R	I	I		
S1-exposed to CA	I		R	R			I		R		R	I			
S1-exposed to CD	R		R	R			I		R		R	I			
S1-exposed to PA	R		R	R	I		I		R		R	I			
S2[d] non-exposed			I	R					R		R	I			I
S2-exposed to TSP	I		R	R					R		R	I			I
S2-exposed to ASC	R		R	R	I		I		R		R	I			I
S2-exposed to CA			R	R					R		R	I			I
S2-exposed to CD	I		R	R					R		R	I			I
S2-exposed to PA			R	R					R		R	I			I

STR, streptomycin; GEN, gentamicin; N, neomycin; RA, rifampicin; AMP, ampicillin; AMC, amoxicillin-clavulanic acid; CF, cephalotin; SXT, sulphamethoxazole-trimethoprim; E, erythromycin, C, chloramphenicol; NA, nalidixic acid; EF, enrofloxacin; CP, ciprofloxacin; TE, tetracycline; FZ, furazolidone; R, resistant strain; I, strain of intermediate susceptibility; no letter, strain susceptible to the antibiotic; exposed strains with increased resistance to antibiotics (relative to unexposed strains) are underlined; exposed strains not having increased resistance to antibiotics are not underlined.

[a] L1, *Listeria monocytogenes* serovar 1/2a (poultry isolate).
[b] L2, *Listeria monocytogenes* serovar 4b (ewe's spinal cord isolate).
[c] S1, *Salmonella enterica* serotype Typhimurium (poultry isolate).
[d] S2, *Salmonella enterica* serotype Enteritidis (poultry isolate).
[e] TSP, trisodium phosphate.
[f] ASC, acidified sodium chlorite.
[g] CA, citric acid.
[h] CD, chlorine dioxide.
[i] PA, peroxyacetic acid.

strains from susceptible to antibiotics (implying that infection due to bacteria will probably respond to that antibiotic) to resistant (implying that infection due to bacteria will probably not respond to that antibiotic).

There have been several laboratory-based investigations describing, for various microbial groups, a possible linkage between the use of sub-inhibitory concentrations of biocides and the acquisition of antibiotic resistance, especially with regard to chlorinated compounds (Russell, Tattawasart, Maillard, & Furr, 1998; Braoudaki, & Hilton, 2004, 2005; Karatzas et al., 2007; Randall et al., 2007; Aiello, 2008). It should be pointed out, however, that clinical levels of resistance were not reached in most reported results, and epidemiological data indicating public health relevance are lacking. Potenski, Gandhi, and Matthews (2003) reported that exposure to acetic acid induced bacterial resistance to multiple antibiotics (tetracycline, chloramphenicol, nalidixic acid and ciprofloxacin). In many studies, similar mechanisms have been implicated in resistance linkage to biocides and antibiotics such as changes in the cell envelope (reduction in porins and changes in LPS and other lipids), multi-drug efflux pumps (able to pump out a wide range of compounds), over-expression of multi-gene components or operons, and the alteration of the target site (SCENIHR, 2008).

Other investigations have failed to make a direct link between biocide exposure and antibiotic resistance (Charnock, 2003; Gradel, Randall, Sayers, & Davies, 2005; Thomas, Russell, & Maillard, 2005; Lear, Maillard, Dettmar, Goddard, & Russel, 2006).

The movement of strains from the category of "sensitive" to that of "resistant" observed for streptomycin, cephalotin and chloramphenicol after exposure to decontaminants is a matter for concern because aminoglucosides (e.g. streptomycin), combined with penicillin, are the drugs of choice for treatment of human listeriosis (Drevets & Bronze, 2008), and cephalosphorins (e.g. cephalotin) are frequently used for treatment of salmonellosis in young patients (Larkin et al., 2004).

The variations in the increases in resistance observed between antibiotics after exposure to decontaminants does not coincide with results from other authors (Potenski et al., 2003), who reported increases in antibiotic resistance of similar magnitude to chloramphenicol, tetracycline, nalidixic acid and ciprofloxacin, after treatment of *Salmonella* strains with chlorine, sodium nitrite, sodium benzoate or acetic acid.

After exposure to ASC strains showed a higher number of increased resistances (13) than when exposed to TSP (7), CA (3), CD (5) or PA (7) (Table 2). These results are not surprising because ASC showed, among the compounds tested, the highest MIC increases (from 1.88 to 2.71-fold) after repeated passages through media containing increasing concentrations of decontaminants (Alonso-Hernando et al., 2009). According to Braoudaki and Hilton (2005) a relationship between lack of susceptibility to biocides and resistance to antibiotics is generally found. The decreases in susceptibility observed for both ASC and antibiotics, which have different modes of action, suggest a non-specific mechanism for resistance (e.g. increased impermeability due to outer membrane adaptation) (Lear et al., 2006).

Major differences were observed between the strains tested, with L2 showing the highest number of increased resistances

(12) compared with L1 (5), S1 (8) and S2 (10). This variability suggests that changes in the pattern of susceptibility to antibiotics after exposure to decontaminants might be strain specific rather than species or genus specific, as reported by other authors (Braoudaki & Hilton, 2005). This makes it extremely difficult to predict how a microorganism might respond to sub-lethal exposure to an antimicrobial agent even if data on related strains exist.

To sum up, the exposure of *L. monocytogenes* and *S. enterica* strains to sub-inhibitory concentrations of poultry decontaminants (especially ASC) increased resistance to various antibiotics. Given the debate over the relationship between antimicrobial treatments for poultry carcasses and the dissemination of strains resistant to antibiotics, these are interesting results. The intra-specific differences observed in antibiotic resistances underline the need to screen a wide range of test strains.

However, it should be emphasized that results in this paper derive from laboratory-based experiments. It is clear that further investigations on the possible development of antibiotic resistance when chemicals are applied directly on poultry carcasses are essential in order to substantiate these findings. Moreover, additional studies should be performed to determine if the increases in resistance observed are of clinical significance.

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