Research Paper

# Biocide tolerance in *Salmonella* from meats in Southern Spain

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## Abstract

*Salmonella* serovars sampled from meat products in Southern Spain (Andalucía) during the period 2002-2007 were analyzed in this study. The serovars most frequently detected (in order) were Typhimurium, Enteritidis, Derby, Anatum and Rissen. Isolates (n = 43) were tested for sensitivity to biocides, including the quaternary ammonium compounds benzalkonium chloride (BC), cetrimide (CT) and hexadecylpyridinium chloride (HDP), and the bisphenols triclosan (TC) and hexachlorophene (CF). The minimum inhibitory concentration (MIC) for the quaternary ammonium compounds was in the range of 25 to 50 mg/L for most isolates, although a few isolates required much higher concentrations, up to 250 mg/L. Bisphenols showed higher inhibitory activity, with a MIC of 2.5 to 25 mg/L. A few isolates showed a "non-wildtype" MIC for TC of up to 250 mg/L. These results indicate a low incidence of tolerance towards quaternary ammonium compounds and triclosan among *Salmonella* from meats and meat products.

**Key words:** *Salmonella*, biocides, serovars, meat, chorizo.

## Introduction

*Salmonella enterica* is an important zoonotic foodborne pathogen and the causative agent of gastroenteritis and typhoid fever (Coburn *et al.*, 2007). In 2012, salmonellosis was still the second most often reported zoonotic disease in humans in the EU accounting for 91,034 confirmed human cases, of which 4,181 occurred in Spain (EFSA-ECDC, 2014). The highest levels of non-compliance with *Salmonella* criteria generally occurred in foods of meat origin which were intended to be cooked before consumption (EFSA-ECDC, 2014). The common reservoir of *Salmonella* is the intestinal tract of a wide range of domestic and wild animals. For that reason, a variety of foodstuffs, including those of both animal and plant origin, can be a source of infection. Transmission often occurs when organisms are introduced in food preparation areas and are allowed to multiply in food, for example because of inadequate storage temperatures, inadequate cooking or cross contamination of ready-to-eat (RTE) foods.

Previously published reports have suggested that *Salmonella* can remain on surfaces and equipment used for handling and washing raw meat and that conventional cleaning and sanitation may fail to eradicate *Salmonella* from such surfaces (McKee *et al.*, 2008; Arguello *et al.*, 2012; Hernández *et al.*, 2013). Biofilm formation is important for the persistence of *Salmonella* in the environment, allowing resistance to different stress factors including biocides (Steenackers *et al.*, 2011). Biocidal compounds are widely used in the food industry for the disinfection of food containers, belts, production plants, to control microbial growth in food and drink, and to decontaminate animal carcasses (McDonnel and Russell, 1999; Cerf *et al.*, 2010). Cationic agents such as benzalkonium chloride and chlorhexidine have been formulated as farm disinfectants (Boddie *et al.*, 1997). Many different biocidal substances are also used for various purposes in the home, as building materials, consumer products or medical devices and on surfaces (SCENIHR, Scientific Committee on Emerging and Newly Identified Health Risks, 2009). Biocide tolerance in

bacteria is becoming a matter of concern because several resistance mechanisms can also accommodate other antimicrobials or are co-selected with antibiotic resistance (Ortega Morente *et al.*, 2013). The increased use of biocides has raised concerns about a possible role in the selection for antibiotic resistant bacteria (Levy, 2002; Russell, 2001; Beier *et al.*, 2004; Ortega Morente *et al.*, 2013). Thus, acquisition of increased tolerance to biocides by zoonotic food-borne bacteria has important implications for public health. The purpose of the present study was to analyze the biocide tolerance of *Salmonella* strains from different types of meat products, including traditionally fermented meats such as chorizo. Initially, isolates of *Salmonella* detected in various meat sources in Andalucía (Southern Spain) during the period 2002-2007 were classified into serovars and then, a subset was selected for a bioide tolerance evaluation.

## Materials and Methods

### Bacterial strains

Isolates were obtained from a total of 112 meat samples that tested positive for *Salmonella* in accordance with the rules of EN/ISO 6579-2002 in official inspections carried out in the Andalucía region during the period 2002-2007. Isolates were serotyped by the "Laboratorio Nacional de Referencia para *Salmonella* y *Shigella* de España" (LNRSSE; Instituto de Salud Carlos III, Majadahonda, Madrid, Spain). Of these isolates, forty-three collected in Jaén (a province in the Andalucía region) were studied further for biocide resistance as described below. Isolates were routinely cultivated on brain heart infusion broth (BHI, Scharlab, Spain) at 37 °C, and stored in cultured broth supplemented with 20% glycerol at -80 °C.

### Determination of biocide tolerance

Overnight cultures were inoculated (1% vol/vol) at approximately $1 \times 10^6$ $\log_{10}$ colony forming units (CFU)/mL in BHI broth supplemented with ten-fold serial dilutions of the following biocides: benzalkonium chloride (BC), cetrimide (CT), hexadecylpyridinium chloride (HDPC), triclosan (TC) and hexachlorophene (CF). The BC commercial solution contained 50% (wt/v) of the active compound. TC and CF were dissolved (10% wt/v) in 96% ethanol. CT and HDPC were dissolved aseptically in sterile distilled water at final concentrations of 1.0%. Biocides were purchased from Sigma-Aldrich (Madrid, Spain). Minimum inhibitory concentrations (MICs) were determined by the broth micro-dilution method in 96-well microplates after 24 h incubation at 37 °C. Growth and sterility controls were included for each isolate. Optical density (OD 595 nm) was determined in an iMark Microplate Reader (BioRad, Madrid). Two independent determinations were conducted for each biocide, and in the case of discordant MIC values, a third repetition was carried out.

## Results and Discussion

Between 2002 and 2007, a total of 112 meat samples in Andalucía were considered positive for *Salmonella* after identification and confirmatory tests for the corresponding 112 isolates by health inspection services (Table 1). The meat products most frequently confirmed as contaminated with *Salmonella* in Andalucía were derived from pork, especially "chorizo", a traditional type of sausage that is slightly fermented and ripened for a variable time period and often consumed without cooking. It has been reported that Enterobacteriaceae (including *Salmonella*) are generally either stable or increase slightly at the beginning of the ripening of chorizo, and then decrease during the late ripening period (Domínguez *et al.*, 1989; Castaño *et al.*, 2002). Previous studies have shown the presence of *Salmonella* in Mexican chorizo (Kuri *et al.*, 1995; Escartín *et al.*, 1999), and an outbreak involving 294 people (of which 73 were confirmed of salmonellosis and 221 were classified as probable cases), associated with the consumption of chorizo in restaurants in Nevada, USA (Nguyen, 2013), was reported in 2013. While poultry products have been traditionally considered as the principal source of *Salmonella* contamination, pork products are also very significant. According to EFSA-ECDC 2012 report (EFSA-ECDC, 2014), the highest levels of positive samples at the slaughterhouse were found in Belgium (10.8%) and Spain (7.8%).

**Table 1** - Sources of the 112 confirmed *Salmonella* isolates from meats and meat products in Andalucía for the period 2002-2007.

| Meat samples | Percentage of isolates (%) | Meat samples | Percentage of isolates (%) |
|---|---|---|---|
| Fresh chorizo | 29.46 | Fresh bovine meat | 1.79 |
| Ripened chorizo | 31.25 | Hamburger meat | 3.57 |
| Extra quality chorizo | 1.79 | Fresh turkey breast | 1.79 |
| Fresh pork meat | 9.82 | Sliced chicken meat | 1.79 |
| Minced pork meat | 2.68 | Roasted chicken | 1.79 |
| Deboned pork meat | 1.79 | Fresh poultry sausages | 4.46 |
| Meat sausages | 1.79 | Others | 1.79 |
| Stuffed meat | 4.46 | | |

The 112 confirmed *Salmonella* isolates belonged mainly to the following serovars (in order): Typhimurium, Enteritidis, Derby, Anatum and Rissen (Table 2). These results agree with the data from EFSA reports that identify *S.* Enteritidis and *S.* Typhimurium as the serovars most frequently associated with human salmonellosis in the EU as well as in the US (EFSA-ECDC, 2014; Centers for Disease Control, 2006). Human *S.* Enteritidis cases are most commonly associated with the consumption of contaminated eggs and poultry, while *S.* Typhimurium cases are mostly associated with the consumption of contaminated pork or beef (EFSA-ECDC, 2014). The subset of 43 isolates from the Jaén province was obtained both during routine inspection and also during a specific control program for all meat vendors in the province, as well as from meat products implicated in salmonellosis. Among these, the Typhimurium serovar was still the most prevalent (Table 2), but the Anatum and Derby serovars were also important, and the Enteritidis serovar was much less prevalent and had the same incidence as the Rissen, Brandenburg or Ohio serovars. According to previous studies, *S.* Typhimurium and *S.* Derby are two of the most frequent *S. enterica* serovars found in pigs across the EU (EFSA, 2010; Matiasovic *et al.*, 2014). While *S.* Typhimurium can colonize many animal species, *S.* Derby is adapted to pigs (Hoelzer, 2011) and contaminated pork is a common source of human *S.* Derby in Europe (Hauser *et al.*, 2011; Kerouanton *et al.*, 2013). The Rissen serovar is also frequently associated with pigs (Vico *et al.*, 2011). *Salmonella* Anatum is most frequently associated with red meat (Fegan *et al.*, 2005; Humphrey and Jørgensen, 2006), but it can also be associated with poultry (Angkititrakul *et al.*, 2005) and pigs (Hoelzer *et al.*, 2011).

In a recent study (Hernández *et al.*, 2013), up to fourteen different serotypes of *Salmonella* were isolated from a free-range pig processing plant (tracking in trucks, lairage, slaughter line and quartering). It was concluded that the processing plant might represent a high risk for the contamination of pork from free-range pigs both prior to and after slaughter, requiring proper cleaning and disinfection operations (Hernández *et al.*, 2013). Effective hygiene control measures coupled with the use of appropriate disinfection regimens are important tools for the control of *Salmonella*

**Table 2** - Relative abundance of serovars among the 112 *Salmonella* confirmed isolates from meat samples in Andalucía during the period 2002-2007, including the 43 isolates from the province of Jaen.

| Serovar | Andalucía (%) | Jaén (%) |
|---|---|---|
| Typhimurium | 34.82 | 41.86 |
| Anatum | 12.50 | 16.28 |
| Enteritidis | 17.86 | 4.65 |
| Derby | 13.39 | 11.63 |
| Rissen | 4.46 | 4.65 |
| Brandenburg | 3.57 | |
| Bredeney | 1.79 | |
| Agona | 0.89 | |
| Ohio | 4.65 | |
| Others* | 10.71 | 11.63 |

*Other serovars for Andalucía include monophasic variant, Gold Coast, London, Nottingham, Ohio, Panama and Senftenberg, as well as London, Give, Nottingham, Panama and Senftenberg for Jaén province.

in food production and processing environments. Because biocides are widely used for disinfection, we wanted to determine the biocide tolerance of *Salmonella* in the available isolates from the province of Jaen. Among the quaternary ammonium compounds tested, benzalkonium chloride inhibited most isolates (86.047%) at 50 mg/L and only one isolate (2.33%) required a much higher concentration of 250 mg/L for inhibition (Table 3). Cetrimide showed an activity similar to that of benzalkonium chloride, although there were more isolates requiring a biocide concentration higher than 50 mg/L. Hexadecylpyridinium chloride also showed the highest activity at 50 mg/L, inhibiting 55.82% of isolates at this concentration. The bisphenol triclosan was the most active of all the biocides tested, inhibiting 79.07% of the isolates at 5 mg/L. However, 9 isolates required higher concentrations of this biocide for inhibition. One isolate had a MIC of 250 mg/L. Another bisphenol, hexachlorophene, was less effective than triclosan at 2.5 mg/L, but none of the isolates tested required a biocide concentration higher than 25 mg/L for inhibition. Altogether, the results obtained show that most investigated isolates of *Salmonella* can be inhibited by relatively low biocide concentrations. For most of the biocides tested,

**Table 3** - Minimum inhibitory concentrations of biocides for *Salmonella* strains from meats. The percentages (%) of isolates inhibited at each biocide concentration are shown.

| | Minimum inhibitory concentrations of biocide (mg/L) | | | | |
|---|---|---|---|---|---|
| Biocide | 2.5 | 25 | 50 | 100 | 250 |
| Benzalkonium chloride | 2.33 | 9.30 | 86.04 | | 2.33 |
| Cetrimide | | 4.65 | 88.37 | 4.65 | 2.33 |
| Hexadecylpyridinium chloride | | 37.20 | 55.82 | 6.98 | |
| Triclosan | 79.06 | 9.30 | 6.98 | 2.33 | 2.33 |
| Hexachlorophene | 51.16 | 48.84 | | | |

there was always a concentration at which most isolates were sensitive. That concentration represents the limit of biocide tolerance for the wildtype population and is also known as the epidemiological cutoff value (ECOFF). In contrast, isolates with a higher ("non-wildtype") MIC should be considered biocide tolerant. Furthermore, one isolate had a high MIC for three different biocides (including all of the quaternary ammonium compounds and triclosan), and two others had a MIC of 100 mg/L for both cetrimide and hexadecylpyridinium chloride. Isolates with a non-wildtype MIC as well as those with tolerance to multiple biocides should be further investigated regarding genetic determinants for biocide tolerance.

Benzalkonium chloride is widely used for sanitizing the surfaces of utensils and instruments in the food industry (Ueda and Kuwabara, 2007; SCENIHR, 2009). Hexadecyl-pyridinium chloride (cetylpyridinium chloride) was approved in 2004 by the US FDA for decontaminating raw poultry at a working concentration not exceeding 0.8% by weight (Food and Drug Administration, 2004). Another common biocide is triclosan, which is now incorporated in a wide range of products such as dishcloths, food boxes, toothbrushes, washing-up liquid and hand-washing gels, plastics, chopping boards, chopsticks, pizza cutters, food storage containers, garbage bags, and kitty litter, among others. Hexachlorophene is often used in soaps and also as a topical anti-bacterial agent (Yazdankhah *et al.*, 2006; SCENIHR, 2009). In conclusion, results from this study indicate that a low percentage of the *Salmonella* isolates investigated can tolerate biocides belonging to the groups of quaternary ammonium compounds (benzalkonium chloride, cetrimide and hexadecylpyridinium chloride) and bis-phenols (triclosan). Therefore, it seems likely that exposure to these biocides could result in the selection of salmonellae able to tolerate higher biocide concentrations. Accordingly, a possible contribution of biocide tolerance to the persistence of *Salmonella* in the food chain should be further investigated. There is a concern that biocide tolerance might co-select for antibiotic resistance determinants in the food chain (reviewed by Ortega Morente *et al.*, 2013). Therefore, further studies should be carried out on the antibiotic resistances of *Salmonella* isolates that have shown a higher tolerance to biocides.

## Acknowledgments

## References

Angkititrakul S, Chomvarin C, Chaita T *et al.* (2005) Epidemiology of antimicrobial resistance in *Salmonella* isolated from pork chicken meat and humans in Thailand. Southeast Asian J Trop Med Public Health 36:1510-1515.

Arguello H, Carvajal A, Collazos JA *et al.* (2012) Prevalence and serovars of Salmonella enterica on pig carcasses slaughtered pigs and the environment of four Spanish slaughterhouses. *Food Res Int* 45:905-912.

Beier RC, Bishoff KM, Poole TL (2004) Disinfectants (biocides) used in animal production: antimicrobial resistance considerations. In Beier RC, Pillani SD, Phillips TD *et al.* (ed). Preharvest and postharvest food safety: contemporary issues and future directions. Blackwell Publishing Professional, Ame, p 227-238.

Boddie RL, Nickerson SC, Adkinson RW (1997) Efficacies of teat germicides containing 0 5% chlorhexidine and 1% iodine during experimental challenge with *Staphylococcus aureus* and *Streptococcus agalactiae.* J Dairy Sci 80:2809-2814.

Castaño A, Garcia Fontán MC, Fresno JM *et al.* (2002) Survival of *Enterobacteriaceae* during processing of Chorizo de cebolla, a Spanish fermented sausage. Food Control 13:107-115.

Centers for Disease Control and Prevention (2006) Multistate outbreak of *Salmonella* Typhimurium infections associated with eating ground beef - United States, 2004. MMWR Morb Mortal Wkly Rep 50:180-182.

Cerf O, Carpentier B, Sanders P (2010) Tests for determining in-use concentrations of antibiotics and disinfectants are based on entirely different concepts: "Resistance" has different meanings. Int J Food Microbiol 136:247-254.

Coburn B, Grassl GA, Finlay BB (2007) *Salmonella* the host and disease: a brief review. Immunol Cell Biol 85:112-118.

Domínguez MC, Gutérrez LM, López A *et al.* (1989) Evolución de los principales grupos de microorganismos durante la maduración del chorizo de León. Alimentaria 26:11-15.

EFSA (2010) Quantitative microbiological risk assessment on *Salmonella* in slaughter and breeder pigs: Final report VLA/RIVM/Food-DTU. (Available online: http://www efsa europa eu/en/scdocs/doc/46e pdf. Accessed May 7, 2014)

EFSA-ECDC (2014) The European Union Summary Report on Trends and Sources of Zoonoses Zoonotic Agents and Food-borne Outbreaks in 2012. EFSA J 12:3547.

Escartín EF, Castillo A, Hinojosa-Puga A *et al.* (1999) Prevalence of *Salmonella* in chorizo and its survival under different storage temperatures. Food Microbiol 16:479-486.

Fegan N, Vanderlinde P, Higgs G *et al.* (2005) A study of the prevalence and enumeration of *Salmonella enterica* in cattle and on carcasses during processing. J Food Prot 68:1147-1153.

Food and Drug Administration (2004) Department of Health and Human Services 21 CFR Part 173 375. Federal Register/Vol 69 No 64/Friday April 2 2004/Rules and Regulations.

Hauser E, Hebner F, Tietze E *et al.* (2011) Diversity of *Salmonella enterica* serovar Derby isolated from pig pork and humans in Germany. Int J Food Microbiol 151:141-149.

Hernández M, Gómez-Laguna J, Luque I *et al.* (2013) Salmonella prevalence and characterization in a free-range pig processing plant: Tracking in trucks lairage slaughter line and quartering. Int J Food Microbiol 162:48-54.

Hoelzer K, Moreno Switt AI, Wiedmann M (2011) Animal contact as a source of human non-typhoidal salmonellosis. Vet Res 42:34.

Humphrey T, Jørgensen F (2006) Pathogens on meat and infection in animals - Establishing a relationship using *Campylobacter* and *Salmonella* as examples. Meat Sci 74:89-97.

Kerouanton A, Rose V, Weill FX *et al.* (2013) Genetic diversity and antimicrobial resistance profiles of *Salmonella enterica* serotype Derby isolated from pigs pork and humans in France. Foodborne Pathog Dis 10:977-984.

Kuri V, Madden RH, Collins MA (1995) Hygienic quality of raw pork and chorizo (raw pork sausage) on retail sale in Mexico City. J Food Prot 59:141-145.

Levy SB (2002) Active efflux, a common mechanism for biocide and antibiotic resistance. J Appl Microbiol 92:65S-71S.

Matiasovic J, Stepanova H, Kudlackova H *et al.* (2014) Immune response of pigs to *Salmonella enterica* serovar Derby and Typhimurium infections. Vet Microbiol 170:284-290.

McDonnel G, Russell AD (1999) Antiseptics and disinfectants: activity action and resistance. Clin Microbiol Rev 12:147-179.

McKee S, Townsend J, Bilgili S (2008) Use of a scald additive to reduce levels of *Salmonella* Typhimurium during poultry processing. Poultry Sci 87:1672-1677.

Nguyen L (2013) *Salmonella* Gastroenteritis Outbreak Among Patrons of Firefly on Paradise Restaurant - Las Vegas Nevada Southern Nevada Health District Interim Report 3 (Available online: http://www southernnevadahealthdistrict org/download/stats-reports/firefly-interim-report-052213 pdf. Accessed on May 7, 2014).

Ortega-Morente E, Fernández-Fuentes MA, Grande-Burgos MJ *et al.* (2013) Biocide tolerance in bacteria. Int J Food Microbiol 162:13-25.

Russell AD (2001) Mechanisms of bacterial insusceptibility to biocides. Am J Infect Contr 29:259-261.

SCENIHR Scientific Committee on Emerging and Newly Identified Health Risks (2009) -Assessment of the Antibiotic Resistance Effects of Biocides January 2009 24-25 (Available online: http://ec europa eu/health/archive/ph_risk/committees/04_scenihr/docs/scenihr_o_021 pdf. Accessed on May 7, 2014) .

Steenackers H, Hermans K, Vanderleyden J *et al.* (2011) *Salmonella* biofilms: an overview on occurrence, structure, regulation and eradication. Food Res Int 45:502-531.

Vico JP, Rol I, Garrido V *et al.* (2011) Salmonellosis in finishing pigs in Spain: prevalence antimicrobial agent susceptibilities and risk factor analysis. J Food Prot 74:1070-1078.

Ueda S, Kuwabara Y (2007) Susceptibility of biofilm *Escherichia coli Salmonella* Enteritidis and *Staphylococcus aureus* to detergents and sanitizers. Biocontrol Sci 12:149-153.

Yazdankhah SP, Scheie AA, Høiby EA *et al.* (2006) Triclosan and antimicrobial resistance in bacteria: an overview. Microb Drug Resist 12:83-90.

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