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Biocide and antibiotic susceptibility of *Salmonella* isolates obtained before and after cleaning at six Danish pig slaughterhouses

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ABSTRACT

Salmonella sp. continues to be one of the most important foodborne pathogens. Control measures in terms of cleaning and disinfection on food production plants are very important for limiting the risk of contaminated food products to reach the consumer. In the last decade concern has arisen that bacteria exposed to disinfectants can develop resistance toward disinfectants and can have a higher risk of developing antibiotic resistance.
The objectives of this study were to examine the prevalence of biocide resistant *Salmonella* sp. in Danish pig slaughterhouses, to evaluate if there was a correlation between susceptibilities to biocides and antibiotics, and to examine if cleaning and disinfection select isolates with changed susceptibility toward biocides or antibiotics. *Salmonella* sp. was isolated from the environment in Danish pig slaughterhouses before and after cleaning and disinfection. The susceptibility toward three different biocides, triclosan and two commercial disinfection products: Desinfect Maxi, a quaternary ammonium compound, and Incimaxx DES, an acetic compound, was determined. We found no resistance toward the biocides tested, but we did find that isolates obtained after cleaning had higher minimum inhibitory concentration (MIC) values toward one of the disinfectants (Incimaxx DES) compared to isolates obtained before cleaning and disinfection. This could indicate selection of strains that are more tolerant, due to the cleaning and disinfection.
Furthermore, we found that there was a weak statistical correlation between MICs toward the biocides and some antibiotics, but no difference in log(MIC)s toward antibiotics between isolates obtained before and after cleaning, nor did we find any difference in the number of resistances of isolates obtained before and after cleaning and disinfection.

1. Introduction

Denmark is among the largest producers of pork in the world with more than 15.5 million pigs slaughtered per year (Anonymous, 2013). In the process of doing so, large quantities of disinfection substances are used on a routine basis to clean the slaughterhouse environment.

Cleaning programs in slaughterhouses are often comprised so that a different disinfection agent is used at least once a week. Disinfection agents may contain quaternary ammonium compounds (QACs), acidic compounds, hydrogen peroxide, or hypochlorite (personal communication, Danish slaughterhouses, 2013). Triclosan (5-chloro-2-(2,4-dichlorophenoxy)phenol) is a phenolic compound, that is used commercially to inhibit bacterial growth and as an antibacterial agent in many products ranging from toothpaste and soap to sportswear and cutting boards. Triclosan is considered bacteriostatic in concentrations below 1000 mg/L and bactericidal in concentrations above (Bailey et al., 2009).

It has been speculated that use of disinfectants and other biocides like triclosan may contribute to the emergence of bacteria more tolerant toward them (Braoudaki and Hilton, 2004; Hegstad et al., 2010; Ortega Morente et al., 2013; Randall et al., 2007). As a number of biocides, e.g. QACs, triclosan, diamidines and chlorhexidine, have been found to be involved in the selection of bacteria with low-level antibiotic resistance (Braoudaki and Hilton, 2004; Whitehead et al., 2011), it is feared that industrial use of biocides may contribute to an increase of antibiotic resistant bacteria from food animals (Alonso-Hernando et al., 2009).

Salmonella is one of the most important foodborne pathogens in Europe with contaminated pork as the fifth most frequent cause of outbreaks (E.F.S.A., 2013). The slaughtering process has in several cases been implicated in the spreading of *Salmonella* in the pork chain (Arguello et al., 2013a; Botteldoorn et al., 2003; Mcdowell et al., 2007; Van Hoek et al., 2012). Cleaning and disinfection are essential to avoid bacterial colonization of the slaughterhouse equipment and limit the risk of cross contamination.

Abbreviations: BPW, buffered peptone water; EFSA, European Food Safety Authority; EUCAST, European Committee on Antimicrobial Susceptibility Testing; MIC, minimum inhibitory concentration; MSRV, Modified Semisolid Rappaport Vassiliadis; PFGE, pulsed field gel electrophoresis; QAC, quaternary ammonium compound.

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The prevalence of biocide resistant isolates of *Salmonella* from Danish pork production has never been investigated. The objectives of this study were to determine *Salmonella* isolation frequency from selected places of the slaughterhouse environment of six Danish pig slaughterhouses and to determine whether common disinfection procedures selected for *Salmonella* that are more tolerant to biocides, and whether this was associated with an increased level of antibiotic resistance.

2. Materials and methods

2.1. Biocides

The commercial disinfection agent Desinfect Maxi (ITW, Novadan Aps) contains didecyl-dimethyl ammonium chloride and belongs to the group of quaternary ammonium compounds (QACs). Incimaxx DES (kind gift of Ecolab, Valby, Denmark) is also a commercial product used for disinfection. It contains acetic acid, hydrogen peroxide, and peracetic acid. Products were prepared in stock solutions of 5% and 20%, respectively using sterile milliQ-water. A stock solution of 50,000 mg/L triclosan (Irgasan, Sigma-Aldrich, Brøndby Denmark) was made in 70% ethanol and from this a solution of 300 mg/L was prepared in sterile MilliQ-water and kept at 5 °C until precipitations had dissolved. The tested biocides and manufacturer's recommended working concentration can be seen in Table 1.

2.2. Sampling

Six pig slaughterhouses in Denmark were included in the investigation. In the majority of the examined slaughterhouses an acidic disinfection agent was used once or twice a week. In only one slaughterhouse, generally using an acidic disinfection agent, a hypochlorite containing disinfection agent was used once a week.

Sampling of all slaughterhouses took place from end of May 2011 until end of February 2012. Samples were collected once a week and were drawn both before and after cleaning and disinfection in three or four consecutive weeks. Sampling was done with a sterile 16-layer cotton swab damped with 10 mL of buffered peptone water (BPW) or saline. Three samples were taken from at least two different places in each slaughterhouse (Table 2). Samples were stored cold (0–10 °C) until they reached the laboratory, and the time between sampling and beginning of analysis varied between one and seven days.

2.3. Isolation and verification of Salmonella

Upon arrival, samples were added 50 mL of preheated BPW (Merck KGaA, Darmstadt, Germany) and incubated overnight at 37 °C for nonselective pre-enrichment. One hundred microliters were spot-inoculated divided in three drops on Modified Semisolid Rappaport Vassiliadis (MSRV) (Oxoid A/S, Roskilde, Denmark) and incubated at 41 °C for 21–27 h. From swarming zones, if present, culture material was streaked onto brilliant green lactose saccharose phenol red agar (Difco, Albertslund, Denmark). Red colonies were classified as presumptive *Salmonella* and five isolates were tested using triple sugar iron (Oxoid A/S, Roskilde, Denmark), Simmons citrate (Becton-Dickinson, BBL, Albertslund, Denmark), lysine decarboxylase (Difco, Albertslund, Denmark), sulfite agar (tryptone yeast extract agar containing 500 mg/L ferric citrate, 500 mg/L sodium sulfite and 100 mg/L potassium permanganate), and *Salmonella* specific antiserum (Statens Serum Institute, Copenhagen, Denmark) (modified from De Zutter et al., 1991). Five isolates from each positive sample showing *Salmonella* specific reactions were stored at −80 °C in 15% glycerol.

2.4. Pulsed field gel electrophoresis

Salmonella isolates were examined using pulsed field gel electrophoresis (PFGE) after digestion of genomic DNA with *Xba*I. A published protocol was adhered to (Ribot et al., 2006). Banding patterns were compared using GelCompar II v. 4.6 (Applied Maths) with a tolerance of 2% and optimization of 1.5%. Isolates with 100% similar profiles under these conditions were assumed to be the same pulsotype. Each pulsotype was assigned a letter in alphabetical order.

2.5. Serotyping of Salmonella spp.

One isolate from each pulsotype was serotyped according to the White–Kauffmann–Le Minor scheme using slide agglutination (Grimont and Weill, 2007). Isolates showing identical PFGE profiles were assumed to be of the same serotype.

2.6. Biocide susceptibility

A two-fold micro-dilution of the biocide was made in 195 μL Müller Hinton broth in a 96-well ELISA plate taking into account the inoculum of 5 μL. Inoculum was prepared from an overnight culture diluted to an $OD_{600} = 0.05$. This gave a final OD in the wells of 0.001 (about 1×10^6 CFU/mL). Plates were incubated at 37 °C for 22–25 h and minimum inhibitory concentration (MIC) was determined as the lowest concentration without visible growth. *Salmonella enterica* serovar Typhimurium strain 4/74 (Wallis et al., 1995) was included in all tests as internal control, and a variation in MIC of one dilution higher or lower was accepted. All MIC determinations were performed in duplicate.

2.7. Antibiotic susceptibility

Antibiotic testing was done using the Sensititre plate GNX2F according to the manufacturer's instructions (TREK Diagnostic Systems Ltd., East Grinstead, UK). Antibiotics and ranges can be seen in Table 3. Isolates were grouped according to their pulsotype, whether they were isolated before or after cleaning, slaughterhouse of origin, and place of sampling. At least one strain from each group was tested. Division into categories of sensitive/resistant was done by the interpretation-limits of clinical breakpoints in the software. The software did not suggest interpretation of colistin, doripenem, and tigecycline. In these cases European Committee on Antimicrobial Susceptibility Testing (EUCAST) suggested breakpoints (Breakpoint table for interpretation of MICs and zone diameters version 2.0) were applied (www.EUCAST.org).

Table 1
Overview of the used biocides and manufacturers' recommended working concentrations.

Disinfectant	Company	Concentrations tested	Working concentration	Active components
Desinfect Maxi	ITW Novadan APS	0.002–1%	1–5% (w/V)	Isopropanol <5% Didecyldimethylammonium chloride 5–15%
Incimaxx DES	Ecolab, Valby, Denmark	0.007–4%	1–2% (V/V)	Acetic acid 30–50% Hydrogen peroxide 5–10% Peracetic acid 2–5%
Irgasan (triclosan)	Sigma-Aldrich, Brøndby, Denmark	0.14–64 mg/L	≤3000 mg/L	Triclosan