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Thermal inactivation of antimicrobial-resistant Gram-positive cocci in chicken meat: D-value and Z-value determinations.

DEAN BERTOLATTI[1], STEVEN J. MUNYARD[2], WARREN B. GRUBB[3] AND COLIN W. BINNS[1]

[1]School of Public Health and [3]School of Biomedical Sciences, Curtin University of Technology, GPO Box U1987Perth Western Australia 6845, [2]The Western Australian Centre for Pathology and Medical Research, Food Hygiene Laboratory

Antimicrobial-resistance in Gram-positive bacteria is reported with increasing frequency in strains isolated from food animals. Their isolation from commercial poultry carcasses and meat products constitute a potential risk that resistant strains or resistance genes might spread to humans via the food chain. As bacterial inactivation by thermal process is a critical control point in the safe preparation of many ready-to-eat foods, it is important to determine the thermal resistance of these organisms. The present study was undertaken to investigate the thermal tolerance (D-values and Z values) of antimicrobial-resistant, Gram-positive cocci in ground chicken meat. The antimicrobial-resistant, Gram-positive cocci for this study were isolated from two poultry processing plants in Western Australia. D-value and Z-value data indicate that these isolates do not exhibit enhanced thermal resistant characteristics. The estimated lethal effect of the cooking process for chicken meat indicates that an internal temperature of 70°C for 2.1 minutes would provide a 7-log reduction of all cell suspensions tested.

Keywords: Poultry, thermal inactivation, antimicrobial resistance, Gram-positive cocci

Glossary: D-value (decimal reduction time): The time in minutes to reduce a population of cells by 90% or 1 log cycle at a specific temperature. Z-value: The temperature increase required to reduce the D-value by a factor of 10. F-value (process lethality): The equivalent heating time at a reference temperature with a specified Z-value.

Introduction

In recent years there has been a dramatic increase worldwide in the number of Gram-positive cocci resistant to one or more antimicrobial groups. During this period antimicrobial-resistant, Gram-positive bacteria have emerged as important pathogens of nosocomially acquired infections (Uttley *et al*.1988, Perez-Trallero and Zigorraga 1995, Jacoby 1996). Antibiotics used in commercial food animal production may impact on human health by contributing to an increase in the prevalence of multidrug-resistant, food-borne pathogens with the potential for increased pathogenicity under specific conditions.

Risk factors of staphylococci and enterococci

Staphylococci produce a variety of local and systemic infections with a range of clinical manifestations. The ability of *S. aureus* to develop resistance to antimicrobials has made the species one of the most important community and nosocomially acquired pathogenic organisms (Mitsuyama *et al*.1997). It remains one of the most frequently reported causes of food-borne intoxication and is a hardy organism, which has a high heat resistance for a non-sporing mesophilic bacterium (Ash 1997).

Enterococci grow and survive under a wide range of environmental conditions including extremes of temperature and salt concentrations. They are found in the intestinal tract and faeces of humans and other animals including food animals (Hardie and Wiley 1997) and may occur in large numbers in food products. Enterococci are of concern because of their increasing importance in nosocomial infections and the increasing numbers of them resistant to glycopeptide antibiotics, such as vancomycin and teicoplanin. While a link between animal and human VRE reservoirs through the food chain has been suggested, the origin and epidemiology of VRE in humans is still unclear (Bates *et al* 1994). The possibility that vancomycin-resistance can be transferred to other Gram-positive bacteria (Noble *et al.* 1992), especially *S. aureus* and coagulase-negative staphylococci has significant implications for human medicine.

Food as a vehicle for transfer of antimicrobial resistance

Current knowledge of the source and the spread of antimicrobial-resistant bacteria in the community are, at best, fragmentary. It is suggested that the use of antimicrobial agents in

animal husbandry might constitute a risk factor in creating an animal reservoir of antimicrobial-resistant bacteria (Tenover and Hughes 1996, Tenover and McGowan 1996). From this reservoir, resistant strains or resistance genes might spread via the food chain. A recent Italian survey of retail outlets reported the isolation of multidrug-resistant enterococci from meat and poultry products, of which a higher prevalence of vancomycin resistance was found in isolates from chicken samples (Pavia *et al.* 2000). The possibility that bacteria from commercial poultry may disseminate antibiotic resistance into the community has important relevance to public health, considering that the annual per capita consumption of chicken meat in Australia is about 27 kg per person (Rural Industries Research and Development Corporation 1998).

Importance of thermal inactivation

Because the thermal inactivation of bacteria is a critical control point in the safe preparation of many ready-to-eat poultry products, defining the safety margins of inactivation of antimicrobial-resistant bacteria is of considerable importance to the food industry and regulators. Although Australia does not have mandatory performance standards that specify a quantifiable reduction in bacterial pathogens for cooked meat and poultry products; the USA Federal Code of Regulations provides a useful reference. Regulation 9CFR381.150 requires that any thermal process used for fully cooked poultry products must be sufficient to cause a 7-D reduction in salmonellae (Code of Federal Regulations 1999). This level of reduction is also applicable to other vegetative cells of bacteria that are potential food pathogens.

The purpose of this study was to investigate the thermal tolerance of antimicrobial-resistant Gram-positive cocci isolated from commercially processed broiler chickens in Western Australia. We studied the thermal resistance in ground chicken meat of: 3 groups of *S. aureus* (comprising composites of 5 and 6 isolates); a composite of VRE comprising two *Enterococcus faecalis* isolates and an *E. gallinarum;* and a methicillin-resistant *S. epidermidis* isolate. Details of the bacterial isolates investigated are presented in Table 1. While previous studies have provided thermal inactivation data for food pathogens and spoilage organisms in chicken products, this is the first study to our knowledge to

investigate thermal inactivation of antimicrobial-resistant bacteria isolated from poultry processing plants.

Materials and methods

Bacterial strains

The staphylococcal and enterococcal isolates used in this study (Table 1) were obtained from two poultry processing plants in Western Australia during 1995-1997 (Bertolatti *et al.* 1996, 1998, Coombs *et al*. 1999). Fourteen of the 16 *S. aureus* isolates selected for this study were from broiler chickens isolated at various points in the processing plant, commencing from the shackling of an incoming live bird through to the processed carcass being packaged. The remaining two isolates were recovered from the defeathering machines in the plants. Susceptibility to antimicrobials and chemicals was determined as previously described (O'Brien *et al*. 1999). All 16 *S. aureus* isolates were resistant to at least four antimicrobials, with all showing resistance to penicillin and to sulphonamides. Five isolates were resistant to erythromycin and lincomycin, and four to tetracycline. The 16 *S. aureus* isolates were tested with the TECRA® visual immunoassay kit (TECRA® International Pty. Ltd., Chatwood, NSW) which detects the presence of any of the enterotoxins A B C_1 C_2 C_3 D and E. Not in methods - Five of the isolates tested positive (Table 1). The *S. aureus* were typed by pulse-field gel electrophoresis (PFGE) of *Sma*1 digested chromosomal DNA (O'Brien *et al*. 1999). The PFGE patterns were scanned on a Fluor-S ™ MultiImager and compared using the Multi-Analyst ® /PC (BIO-RAD Laboratories). Isolates were grouped according to PFGE patterns. Group A was a heterogeneous group of unrelated patterns whereas groups B and C comprised isolates with related PFGE patterns. The isolates in group B had 68%-94% similarity while those in group C had 64%-97% similarity to each other. Group B isolates only had 40% similarity with those in group C. Two isolates within group A were closely related to isolates within Group C, but other isolates within this group had little similarity with other isolates (34%-42% similarity).

Three VRE isolates studied (Table 1) were two *E. faecalis* (WBG 9171 and WBG 9172) and one *E. gallinarum* (WBG 9213). All the isolates contained the *vanA* gene and in addition WBG 9213 contained the *vanC* gene (Coombs *et al*. 1999).
A *S. epidermidis* isolate (Table 1) which gave a positive PCR for *mecA* (O'Brien *et al.* 1999) was also studied.

Inoculum preparation

The preparation of cell suspensions was adapted from the procedure described by Blackburn *et al*. (1997). Tryptone soya broth (TSB, Oxoid, Basingstoke, England) was inoculated with the test organisms and incubated at 37°C for 24 hours. The culture was diluted in 0.1% peptone to produce a concentration of about 10 cfu/ml and 1 ml was transferred to 100 ml of TSB, which was incubated at 37°C between 16-19 hours. The 100 ml culture was centrifuged (IEC Centra® MP4R, IEC International Equipment Company, Massachusetts, USA) at 1400 x g for 20 minutes at 21°C and resuspended in 40 ml TSB. The inoculum suspension was enumerated by spiral plating (Don Whitley, Scientific Ltd., West Yorkshire, UK) on plate count agar (PCA, Oxoid, Basingstoke, England) plates and incubated at 37°C for 24 hours.

Ground chicken

Chicken meat used for inoculation was from two ready-to-cook broiler chickens obtained directly from a processing plant. The broiler chickens were transported to the laboratory under refrigerated conditions and on arrival were immediately aseptically deboned in a laminar flow cabinet. The chicken meat was then ground twice through a 4mm-diameter grinder plate in a Hobart N-50G mixer with chopper attachment (Hobart Corporation, Ohio, USA). Approximately a 160g portion of the ground chicken meat was placed in a sterile Whirl-Pak® bag (Nasco Whirl-Pak, Wisconsin, USA) and stored at 4°C until required for proximate analysis. Meat samples for thermal inactivation experiments were weighed (≈60g) and placed into 120 ml sterile polystyrene containers and stored at -20°C.

Proximate analysis

The ground chicken meat samples were analyzed in triplicate for moisture, petroleum ether-extractable lipid, and protein (Kjeldah) content using AOAC methods (AOAC 1990).

Thermal inactivation

For thermal inactivation experiments the *S. aureus* isolates were grown separately and then combined in approximately equal portions to form five and six-isolate composites. Likewise the three VRE isolates were combined to provide a three-isolate composite. The methicillin-resistant, coagulase-negative *S. epidermidis* was examined by itself.

The frozen chicken meat samples were thawed and viable counts determined aerobically by spiral plating (Don Whitley, Scientific Ltd., West Yorkshire, UK). The average of three 40g samples was 3.2×10^2 CFU/g.

Thermal death time determinations were adapted from the methods of Blankenship and Craven (1982), Ahmed *et al*. (1995) and Blackburn *et al*. (1997). Forty-gram portions of raw ground chicken meat were inoculated with 4 ml of the selected bacterial cell suspension and mixed for five minutes in a Seward 400 stomacher (Seward Ltd., London, UK). Two-gram Samples of the inoculated meat were weighed into sterile Whirl-Pak® bags and spread out in a thin layer in the lower third of the bags. The bags were sealed and the lower two-thirds immersed for various times in a shaking water bath equilibrated at 60, 65 or 70°C. Temperatures were measured with RTD (PT 385) probes (Temp Controls, Leichhardt, NSW.) connected to a Datataker DT500 data logger (Data electronics, U.S.A., Inc.). One probe was placed in the bag containing the chicken meat and one in the water bath. Bags were removed from the water bath after heating for given dwell times, and immediately mixed with 18 ml of room temperature sterile 0.1% peptone by mixing for 2 min in a Seward 400 stomacher. The samples were left at room temperature for 90 min to allow recovery of heat-injured cells. Serial 10-fold dilutions were then made in sterile 0.1% peptone and viable counts performed in duplicate by spreading 0.1ml of each dilution onto tryptone soya agar (TSA, Oxoid, Basingstoke, England) plates. Colonies were counted after incubation for 48hours at 37°C. All thermal death time experiments were done in triplicate.

Determination of D values and Z values

To calculate a single regression equation at each temperature for the bacteria tested, survivor curves were obtained by plotting the averages of the three trials (mean $\log_{10}$ CFU/g) versus heating time (minimum of four dwell times). Linear regression using SPSS PC – V9.0 (SPSS Inc., Chicago, Illinois) produced a correlation coefficient and an estimate of the intercept and slope of the straight line for each temperature. The D-values (negative reciprocal of the slope) were calculated from the resulting regression equations. Tailing values in survivor curves were included in calculating D-values. The Z values were estimated from thermal death time curves by regression $\log_{10}$ D values versus heating temperatures. Data on Z values were analysed using SPSS.

Results and discussion

Proximate analysis

Proximate analysis of the ground chicken meat found that the moisture, fat and protein content was 66.6%, 15.9% and 14.7% respectively, the pH was 6.41.

Survivor curves

Survivor curves for the 60, 65, and 70°C trials for the three *S. aureus* groups, the VRE group and the *S. epidermidis* are given in Fig. 1. In all cases, however, a slight tailing of the survivor curves was observed, with low numbers of cells surviving longer than the calculated D values. Humpheson *et al.* (1998) suggested that survivor curves with tailing generally represent a mix of two species or strains with different heat resistance. The straight portion of the curve mainly describes the destruction of the more sensitive cells, while the tailing describes the death of the more resistant microorganisms (Xiong *et al.*1998). It is likely that the slight tailing observed in this study indicates that the population of cells varied in their heat resistance.

D-values

D-values were determined by regression analysis of the survival data for each temperature. Summaries of regression statistics and D-values for the *S. aureus* groups, the three isolate VRE composite and *S. epidermidis* are listed in Table 2.

The regression mean $\log_{10}$ cfu/g (surviving viable cells) versus heating time in minutes was linear for all *S. aureus* groups ($P < 0.05$). The D-values of *S. aureus* groups A, B and C when heated to 60, 65 and 70°C are 7.01, 6.65 and 7.11; 0.70, 0.54 and 0.69; 0.24, 0.25 and 0.23 minutes, respectively. Groups A and C were slightly more heat resistant at 60 and 65°C, but at 70°C there was little difference in resistance between groups with a range of 0.23-0.25 minutes. The goodness of the fit of survival curves was assessed using the correlation coefficient (R-square) between the dependent variable (mean $\log_{10}$ cfu/g) and the independent variable (duration of heating in min.). Linear regression analysis of thermal inactivation data gave a good fit with R-square values > 89% for all *S. aureus* groups at each heating time. $D_{60°C}$ values found in this study are within the range of those reported by Bean and Roberts (1975), where the $D_{60°C}$ for a meat macerate at pH 6.5 was between 4.61 and 9.62 minutes.

While the $D_{60°C}$ value of 7.35 minutes for the VRE cell suspension was the highest value obtained for all trials, it did, however, also provide the least D-value correlation with an R-square value of 83.9%. Conversely the $D_{60°C}$ value of 5.52 minutes for *S. epidermidis* was the lowest.

There was little difference between all bacterial cell suspensions tested at 70°C as the $D_{70°C}$ values ranged from 0.23 to 0.30 minutes indicating that there is little difference in the level of susceptibility to heat in chicken for the organisms investigated.

Z-values

For all cell suspensions over the temperature range studied (60, 65 and 70°C) there was an approximately linear relationship between the logarithm of the predicted D-value and temperature, with R-square values > 90%. The Z-values and regression statistics are presented in Table 3. The Z-values for the *S. aureus* groups were very similar, ranging from 6.66 to 7.04°C. Z-values of 7.46°C and 7.24°C were obtained for *S. epidermidis* and the VRE group respectively.

Lethal process (F-value) estimates

As bacterial inactivation by thermal process is a critical control point in the safe preparation of fully cooked poultry products, it is important that the lethal effect of the cooking process in reducing these antimicrobial-resistant organisms is established. Table 4 provides estimates of F-values required for achieving a 7-D lethality in chicken meat from the $D_{70°C}$ values obtained in this study. The estimated F-values at 70°C ranged from 1.61 to 2.10 minutes, with the enterococcal group requiring slightly longer to achieve a 7-log reduction. The USA require that any thermal process used for fully cooked poultry products must be sufficient to cause a 7-D reduction in salmonellae and other vegetative bacterial cells that are potential food pathogens (Code of Federal Regulations 1999). Where no mandatory performance standards are specified requiring quantifiable microbiological pathogen reduction for cooked poultry products, then these standards provide a useful reference in establishing critical limits for this critical control point.

Conclusion

Heat resistance at 60, 65 and 70°C was determined in samples of ground chicken meat for all cell suspensions tested. There was little difference in heat resistance between the bacterial suspensions tested at 70°C as the $D_{70°C}$ value ranged from 2.3 to 3.0 minutes. D-value and Z-value data indicate that these isolates do not exhibit enhanced thermal resistant characteristics. The effect of temperature on the survival of *S. aureus* isolates in chicken meat in our trials is similar to that reported in other laboratory studies of *S. aureus* in meat macerates. Data estimating the lethality of the cooking process for chicken meat indicates that an internal temperature of 70°C for 2.1 minutes would provide a 7-D reduction of all bacterial cell suspensions tested. It is evident that the heat treatment significantly reduces the risk of cooked chicken constituting a potential vehicle for the transfer of resistant bacteria via the food chain. However, such risks as cross contamination post cooking and the potential for foodhandlers to be colonised by these antimicrobial-resistant organisms cannot be underestimated. Effective management of these risks requires an organized

HACCP system complimented by the application of Good Hygiene Practices (GHP) or Good Manufacturing Practices (GMP).

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Table 1: Bacterial isolates

Groupings Tested	Isolates	Source of Isolation	Antimicrobial Resistance	Staphylococcal Enterotoxin Production
	S. aureus *isolates*			
	1P4	C (throat)	P,S,T,S3,Cd,W	-ve
	1P5	DFM	P,S,T,S3,Cd	-ve
A	4P38	*C (under wing)	P,S,S3,Cd,W	-ve
	6P73	C (throat)	P,S,S3,Cd	+ve
	7P80	C (cloacal)	P,E,L,S3,Cd,W	-ve
	S. aureus *isolates*			
	6P061	*C (under wing)	P,E,L,W,T,S3,Cd,Eb	-ve
	6P064	C (under wing)	P,S,S3,Cd,Eb	+ve
B	7P79	C (cloacal)	P,S,E,L,T,S3,Cd,Eb,W	-ve
	8P126	DFM	P,S3,Cd,W	-ve
	8P128	C (under wing)	P,S3,Cd,Eb	-ve
	S. aureus *isolates*			
	3P23	C (neck skin)	P,S,E,L,S3,Cd	-ve
	6P68	DFM	P,S3,Cd,Eb	+ve
	6P070	C (throat)	P,S3,Cd,Eb	-ve
C	6P72	C (throat)	P,S3,Cd,W	-ve
	7AP86	C (cloacal)	P,S3,Cd,Eb	+ve
	7AP090	C (neck skin)	P,S3,Cd,Eb	+ve
	Enterococci			
	WBG9171	C (under wing)	V(*vanA*), Tc	
VRE	WBG9172	C (neck)	V(*vanA*),Tc (*vanA*)	
	WBG9213	C (cloacal)	V(*vanA* & *vanC*),Tc	
	S. epidermidis			
	Isolate 19A 1	C (throat)	M(*mecA*),P,E,L,T,S,Asa,Eb	

Abbreviations:
P, penicillin; S, streptomycin; T, tetracycline; S3, sulphamethoxazole; Cd, cadmium; W, trimethoprim; E, erythromycin; L, lincomycin; Eb, ethidium bromide; V, vancomycin; Tc, teicoplanin; M, methicillin; Asa, arsenate; C, broiler chicken; DFM, defeathering machine; * = live birds

Table 2: D-values and regression parameters obtained from survivor curves for *Staphylococcus aureus* Groups A, B and **C,** the three isolate VRE composite and *Staphylococcus epidermidis* in raw ground chicken meat.

Temperature (^{0}C)	Regression Parameters				D-value (min)
	Slope	Y intercept Log cfu/g	Correlation co-efficient	R^2	
Staphylococcus aureus					
Gp A					
60	-0.1426	8.1740	-0.9922	0.9845	7.01
65	-1.4257	7.7364	-0.9684	0.9378	0.70
70	-4.2298	7.3819	-0.9473	0.8975	0.24
Gp B					
60	-0.1504	7.8580	-0.9637	0.9288	6.65
65	-1.8406	7.7924	-0.9863	0.9728	0.54
70	-3.9572	7.2823	-0.9495	0.9017	0.25
Gp C					
60	-0.1406	7.8780	-0.9700	0.9409	7.11
65	-1.4384	8.0008	-0.9853	0.9709	0.69
70	-4.3730	7.2358	-0.9447	0.8926	0.23
VRE Isolates					
60	-0.1360	8.2965	-0.9158	0.8388	7.35
65	-1.4394	8.0176	-0.9443	0.8918	0.69
70	-3.2800	7.8533	0.9465	0.8960	0.30

Staphylococcus epidermidis					
60	-0.1809	7.8092	-0.9744	0.9496	5.52
65	-2.0960	7.5500	-0.9541	0.9104	0.48
70	-3.9642	7.5065	-0.9589	0.9195	0.25

Table 3: Z-values and regression statistics for *Staphylococcus aureus* groups A, B and C, VRE and S*taphylococcus epidermidis* in raw ground chicken meat at 60, 65 and 70^0C.

	Regression Parameters				Z-value (^{0}C)
	Slope	Y intercept Log_{10} D-value	Correlation co-efficient	R^2	
S. aureus A	-0.1460	9.5133	-0.9794	0.9594	6.84
S. aureus B	-0.1420	9.2167	-0.9576	0.9170	7.04
S. aureus C	-0.1500	9.7633	-0.9805	0.9615	6.66
VRE Group	-0.1380	9.0300	-0.9639	0.9292	7.24
S. epidermidis	-0.1340	8.6533	-0.9503	-0.9032	7.46

Table 4: Estimated F-values to achieve a 7-D reduction in chicken meat at 70°C.

Organisms	T Ref (^{0}C)	Z(^{0}C)	D (min)	Log Reduction	Required F-value
S. aureus Gp A	70	6.84	0.24	7	1.68
S. aureus Gp B	70	7.04	0.25	7	1.75
S. aureus Gp C	70	6.66	0.23	7	1.61
VRE Group	70	7.24	0.30	7	2.10
S. epidermidis	70	7.46	0.25	7	1.75