Correlation analysis of heat stability of veterinary antibiotics by structural degradation, changes in antimicrobial activity and genotoxicity

M.K. Hsieh[1], C.L. Shyu[2], J.W. Liao[3], C.A. Franje[2], Y. J. Huang[21], S.K. Chang[4], P.Y. Shih[2], C.C. Chou[2]

[1]Graduate Institute of Microbiology and Public Health, College of Veterinary Medicine, National Chung-Hsing University, Taichung, Taiwan
[2]Department of Veterinary Medicine, College of Veterinary Medicine, National Chung-Hsing University, Taichung, Taiwan
[3]Graduate Institute of Veterinary Pathobiology, College of Veterinary Medicine, National Chung Hsing University, Taichung, Taiwan
[4]School of Veterinary Medicine, National Taiwan University, Taipei, Taiwan

ABSTRACT: The relationship between the structural degradation of veterinary antibiotics, their antimicrobial activity, and possible mutagenicity after heating have not been well investigated sequentially. This study aimed to evaluate the heat stability of 14 veterinary antibiotics under a short-term heating scenario by characterization of their structural degradation and their relationship to resultant changes in antimicrobial activity. Mutagenicity was also examined in four representative antibiotics after 15-min-heat treatments at two temperatures (100 °C and 121 °C). Differential heat stabilities of antibiotics between drug classes, between temperature levels, and among the same class of drugs were discovered. Heat treatment resulted in the reduction of the main peak and the production of new peaks in certain antibiotics, contributing to minimum inhibitory concentration increases of 2- to 1024-fold. Ranking of heat stability by antibiotic classes at 121 °C was highest for sulfonamides, followed by lincomycin, colistin, tetracyclines and β-lactams while at 100 °C sulfonamides equaled lincomycin and and was greater than colistin but variability was observed within different tetracyclines and β-lactams. Correlation analysis suggested that except for doxycycline (DC), structural degradation of the drugs was in good agreement with the reduction in antimicrobial activity, suggesting that degradation also diminished antimicrobial activity. Furthermore, the markedly variable heat stabilities within the classes of tetracyclines and β-lactam antibiotics highlighted the fact that heat stability within these two classes can be very different despite their structural similarity; hence, it is not appropriate to predict heat stability simply by antibiotic class. Mutagenicity (Ames) tests on heated chlortetracycline (CTC) resulted in 2- to 6-fold revertant changes in *Salmonella typhimurium* TA98 and TA100. The combined results suggest that correlation analysis of structural degradation and antimicrobial activity offers dual evaluation of a drug's heat stability but gives little advantage over assessment of the resultant toxicity.

Keywords: capillary electrophoresis; minimum inhibitory concentration; mutagenicity; Ames test; thermodegradation

The extensive use of antibiotics as therapy, prophylaxis and growth promotion in food-producing animals raises concern of the occurrence of antibiotic residues in edible tissues after slaughter (Anadon and Martinez-Larranaga, 1999). Antibiotic residues in food have been linked to growing public health concerns over the spread of antibiotic-resistant microorganisms, human allergic reactions and imbalances in intestinal microflora. Moreover, their presence may affect fermentation processes in food production industries (Mourot and Loussourorn, 1981).

For the past 50 years, many researchers have been interested in evaluating whether antibiotic residues can be destroyed by cooking procedures,

pasteurization, or canning processes (Ibrahim and Moats, 1994; Rose et al., 1995; Isidori et al., 2005; Hassani et al., 2008). Traditionally, heat stabilities of antibiotics have been studied based on either the evaluation of the decrease in antimicrobial activity or by specific chromatographic analysis of change in concentration after heat treatments. Relatively few studies have been carried out using both microbiological and chemical analyses in evaluating the heat stability of veterinary drug residues (Franje et al., 2010). Moreover, whether or not the heating of these compounds and any structural changes generated result in altered genotoxicity that could contribute to the mutagenicity of bacteria remains unclear.

Previous studies have suggested that sulfamethazine (SMZ) (Rose et al., 1995; Papapanagiotou et al., 2005) oxacillin (OXA), chloramphenicol, aminoglycosides, quinolones, clindamycin, novobiocin, trimethoprim, vancomycin, and azlocillin are heat-stable (Traub and Leonhard, 1995) while oxytetracycline (OTC) (Hassani et al., 2008) and erythromycin were shown to be heat-labile. On the other hand, several β-lactams such as penicillin (PCN) G, ampicillin (AMP) and amoxicillin (AMX) appear partially heat-labile (Traub and Leonhard, 1995). Antibiotics of the same class were also reported to have different heat stabilities depending on different types of matrices and heating treatments involved (Kitts et al., 1992; Rose et al., 1996; Franje et al., 2010). Most of the findings and the conclusions drawn were summarized by Moats (1999). On the other hand, most heat stability studies evaluated the degradation of parent drugs with few studies carried out on the possible production of toxic breakdown products (Gratacos-Cubarsi et al., 2007; Franje et al., 2010).

In this study, the heat stabilities of 14 common veterinary drugs encompassing five different classes were evaluated employing both microbiological and electropherographic methods to evaluate the correlations between these two assessments. The production of possibly toxic degradation products after the heating of selective antibiotics was further attempted through the Ames test. The Ames Salmonella assay is a rapid, reliable and economical method to screen compounds with potentially harmful genetic activity (McCann et al., 1975; Maron and Ames, 1983). The Ames test was used to evaluate the mutagenicity of several antibiotics and it was shown that sulfamethoxazole (SMX), ofloxacin, lincomycin (LIN) (Isidori et al., 2005),

norfloxacin (Arriaga-Alba et al., 1998), and quinolones (Gocke, 1991) were mutagenic, while the new derivative of anthracycline (WP903) also demonstrated a strong genotoxic action (Chlopkiewicz et al., 2005). However, very few studies have focused on the mutagenicity/genotoxicity of antibiotics after heat treatments. The results of this study should facilitate the understanding of the effects of heat treatment on different types of antibiotics and to evaluate the effectiveness of using both electropherographic and microbiological assays in assessing the heat stability of drugs.

MATERIAL AND METHODS

Study design

Fourteen different antibiotics from major antibiotic classes, including tetracyclines, β-lactams, sulfonamides, colistin and lincomycin were evaluated for their structural integrity after 15 min thermal treatments in water at two temperatures (100 °C and 121 °C) and at two concentrations (50 μg/ml and 200 μg/ml). Heat stability was investigated through evaluation of the qualitative and quantitative electropherographic profiles, UV-PDA spectrometry (200–450 nm), and antimicrobial activity against three test bacteria *Escherichia coli* (*E. coli*, ATCC 25922), *Staphylococcus aureus* (*S. aureus*, ATCC 29213), *Bacillus subtilis* (*B. subtilis*, ATCC 6633). The Ames test for reversibility was applied to selective drugs. For the purpose of the study, a new peak was conveniently defined as a peak in which the area was greater than 10% of the main peak area (non-heated). The degradation of the drug was evaluated by quantifying the reduction in main peak area as well as the appearance of new peak numbers and new peak areas after heating. Data (presented as mean ± SEM) were evaluated for statistical differences among the 14 drugs at different concentrations and temperatures using analysis of variance (ANOVA, SAS 6.12 for Windows; SAS Institute, Cary, NC, USA). Statistical differences were set at $P < 0.05$.

Antibiotics and chemical compounds

The standards for chlortetracycline hydrochloride (CTC), DC, OTC, TC, AMP, AMX, cloxacillin sodium salt (CLO), dicloxacillin sodium salt (DIC),

OXA, colistin sulfate salt (COL), LIN, SMX, and SMZ were all purchased from Sigma-Aldrich (St. Louis, MO, USA) while PCN-G was obtained from Fluka Chemical Co. (Buchs, Switzerland). Sodium dodecyl sulfate, sodium hydroxide, methyl-β-cyclodextrin, sodium borate, sodium phosphate dibasic were obtained from Sigma-Aldrich (St. Louis, MO, USA). Triethanolamine and 2-propanol were supplied by Merck (Darmstadt, Germany). Ethylenediaminetetraacetic acid was supplied by Sigma Chemical Co. (St. Louis, MO, USA). Methanol and HPLC grade water were obtained from Tedia Company, Inc. (Fairfield, OH, USA). Phosphoric acid (Union Chemical Works Ltd., Hsinchu, Taiwan, R.O.C.), sodium phosphate monobasic, anhyd. (YAKURI Pure Chemicals Co., Ltd., OSAKA, Japan), and sodium carbonate (Wako Pure Chemical Industries Ltd., Japan) were all purchased.

Sample preparation

Stock solutions at a concentration of 1 mg/ml were prepared by dissolving each antibiotic in double distilled water (DDW). Working standard solutions at concentrations of 50 and 200 μg/ml (ppm) for each antibiotic were prepared by appropriate dilution of the stock solutions with DDW. For thermal treatments, all working solutions in 10 ml vials were heated for 15 min either immersed in a water bath (Hipoint, Kaohsiung, Taiwan) at 100 °C or autoclaved at 121 °C. Samples of tetracyclines (CTC, DC, OTC, TC) were prepared in amber-colored containers and all samples were prepared and analyzed fresh to avoid any possible degradation caused by prolonged storage.

Capillary electrophoresis (CE)

The CE analyses were based on previously reported methods for tetracyclines (Mamani et al., 2006), β-lactams (Hows et al., 1997), COL and LIN (Kang et al., 2000), and sulfonamides (Fuh and Chu, 2003). Briefly, CE analysis was performed in a Beckman P/ACE 5500 system (Beckman Instruments, Fullerton, CA, USA), coupled to a photodiode array (PDA) detector, and System Gold® software was used for data collection and analysis. Separations were carried out in an uncoated fused-silica capillary (Beckman, Fullerton, CA, USA) of 50 μm internal diameter, with 47 cm total length (effective length

40 cm), and a capillary temperature of 23 °C. UV detection was performed at 270 nm, 205 nm, 192 nm and 205 nm for tetracyclines, β-lactams, COL/LIN, and sulfonamides, respectively. Tetracyclines and β-lactams were separated at 18 kV while COL/LIN and sulfonamides were separated at 30 kV and 25 kV, respectively.

Minimum inhibitory concentration (MIC)

MIC tests were performed based on the reference broth liquid microdilution method described by the Clinical and Laboratory Standards Institute (CLSI) M7-A7 (CLSI, 2008). Briefly, a serial two-fold dilution of each antibiotic was made in 96-well flat-bottom microdilution plates (Becton Dickinson Labware, Franklin Lakes, NJ, USA) using sterile MHB (Becton Dickinson, Sparks, MD, USA) to obtain the final drug concentration ranges of 0.048–50 μg/ml. *Staphylococcus aureus* (ATCC 29213), *Escherichia coli* (ATCC 25922) and *Bacillus subtilis* (ATCC 6633) purchased from The Food Industry Research and Development Institute (FIRDI, Hsinchu, Taiwan) were subcultured for 18 h in tryptic soy agar (Acumedia Manufacturers, Inc., Lansing, MI, USA). Bacterial suspensions at 1×10^8 CFU/ml were prepared in sterile saline (0.9% NaCl) and adjusted to a final inoculum of 1×10^6 CFU/ml. Microdilution plates containing 100 μl of two-fold serial dilutions of antibiotics in each well were inoculated with 100 μl of the final inoculum yielding a final test concentration for each bacterium of 5×10^5 CFU/ml. Following inoculation, the microdilution plates were incubated at 37 °C for both *E. coli* and *S. aureus* and at 30 °C for *B. subtilis* in an ambient air incubator. The OD values were determined after 18 h with a spectrophotometer at a wavelength of 590 nm. For each drug and treatment, the experiment was carried out in duplicated wells for three plates (*n* = 3). The MIC was defined as the lowest antimicrobial concentration that completely inhibited bacterial growth. All tested antibiotics except for the two sulfonamides which have less than 4% degradation were evaluated for their MIC changes after heating.

Ames/mutagenicity test

The mutagenicity of selected heated antibiotics was assayed by the Ames test with the standard

plate incorporation technique as previously described by Maron and Ames (1983) and revised by Mortelmans and Zeiger (2000). *Salmonella typhimurium* strains TA98 (National Institute of Technology and Evaluation; NBRC 14193) and TA100 (NBRC 14194) purchased from FIRDI were used as tester strains. A mixture of 0.1 ml antibiotic sample, 0.5 ml S9 rat liver microsomal enzyme (Lot # 1452, Moltox Inc., USA) homogenate, 0.2 ml histidine/biotin and 0.1 ml of fresh culture of tester strains (approximately 1×10^8 CFU/ml) was added to a tube containing 2 ml of molten top agar (contained 0.75% agar and 0.5% NaCl). The tube was gently vortex-mixed and plated on top of the glucose minimal agar plate. Antibiotic samples (autoclaved CTC, DC, AMX and DIC) were tested in triplicates with and without metabolic activation (S9 mix). Diagnostic mutagens sodium azide (10 μg /ml), 4-nitroquinolone-N-oxide (10 μg/ml) and 2-anthramine (50 μg /ml), all obtained from Sigma-Aldrich Co. (St. Louis, MO, USA) were used as positive controls. The number of revertant colonies was scored after incubation at 37 °C for 48 h. The compound was considered a mutagen if the tested sample produced a response, which was at least twice as high as the one found with the negative control (Mortelmans and Zeiger, 2000).

RESULTS AND DISCUSSION

Structural degradation by peak area

Traditionally, the heat stability of antibiotics is evaluated either by quantitative reduction in drug concentrations or by decreasing antimicrobial activity against susceptible microbes after heat treatments. Employing both microbiological and chemical assays to assess the structural integrity and the associated antimicrobial activity of antibiotics after heating is seldom done. In this study, multiple parameters such as the electropherographic profiles, main peak reduction, new peak and new peak area production, changes in total peak area (percentage main peak reduction plus percentage new peak area increase), and changes in ultraviolet (UV) spectrometry were utilized to assess the structural degradation of antibiotics after heat treatments. The antimicrobial activities of the heated drugs were further assessed through their MIC's and correlations of both the CE and MIC results were made. Furthermore, the presence of
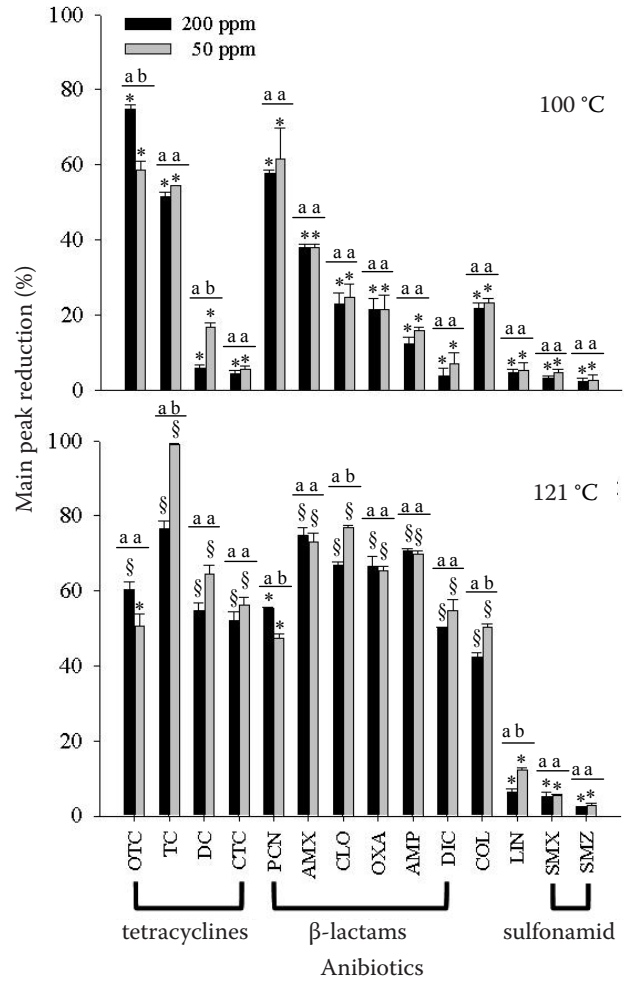

Figure 1. Percentage reduction of main peak area after 15 min boiling or autoclaving of antibiotics in water; different symbols (* and §) denote significant difference between temperatures, different letter (a, b) and bar denote significant difference between concentrations

mutagenicity after heating in selected antibiotics was investigated.

In the studied concentration range (50–200 ppm), the changes in peak area were linearly proportional to the changes in mother drug concentration providing a rational basis for using peak area as reliable estimation of concentration change. The results indicated that the initial concentrations of all tested antibiotics diminished to variable degrees after heat treatments (Figure 1). These results are generally in good agreement by drug classes with previous studies (Rose et al., 1996; Moats, 1999; Podhorniak et al., 1999; van Egmond, 2000; Kuhne et al., 2001a,b; Lolo et al., 2006) confirming that thermal treatments may reduce the concentration of veterinary drug residues in foods and thereby might decrease possible pharmacological and/or toxic effects of these

compounds. Higher percentage reductions were apparent at higher temperatures showing up to 99% reduction at 121 °C in contrast to 54.4% at 100 °C for TC and most other antibiotics (Figure 1), indicating that the degree of reduction was associated with heating temperature. Some exceptions could be found at PCN G and OTC where similar degradation was noted at both temperatures. Higher reductions in antibiotics obtained with heating at higher temperature were also shown in other studies (O'Brien et al., 1981; Ibrahim and Moats, 1994; Rose et al., 1996). As can be observed, the sulfonamides and LIN were considered thermotolerant while the tetracyclines and β-lactams showed variable heat stability characterized by greater than 50% degradation for TC/OTC versus less than 20% for

DC/CTC, and 40-60% degradation for PCN/AMX versus only 20% degradation for DIC/AMP at the temperatures tested. The marked heat stability of sulfonamides at the tested temperatures is supported by Papapanagiotou et al. (2005) and Rose et al. (1995) who used a similar method to analyze SMZ. On the other hand, the notabll variable heat stabilities within the class of tetracyclines and β-lactams antibiotics highlight the fact that the heat stability of these two classes can be very different despite structural similarity; therefore, it is not appropriate to predict heat stability simply by class of antibiotics. This is further supported by the fact that SMZ, SMX, LIN, DIC and CTC, which all have less than 15% reduction, belong to four different classes of antibiotics. Representative structural degradation
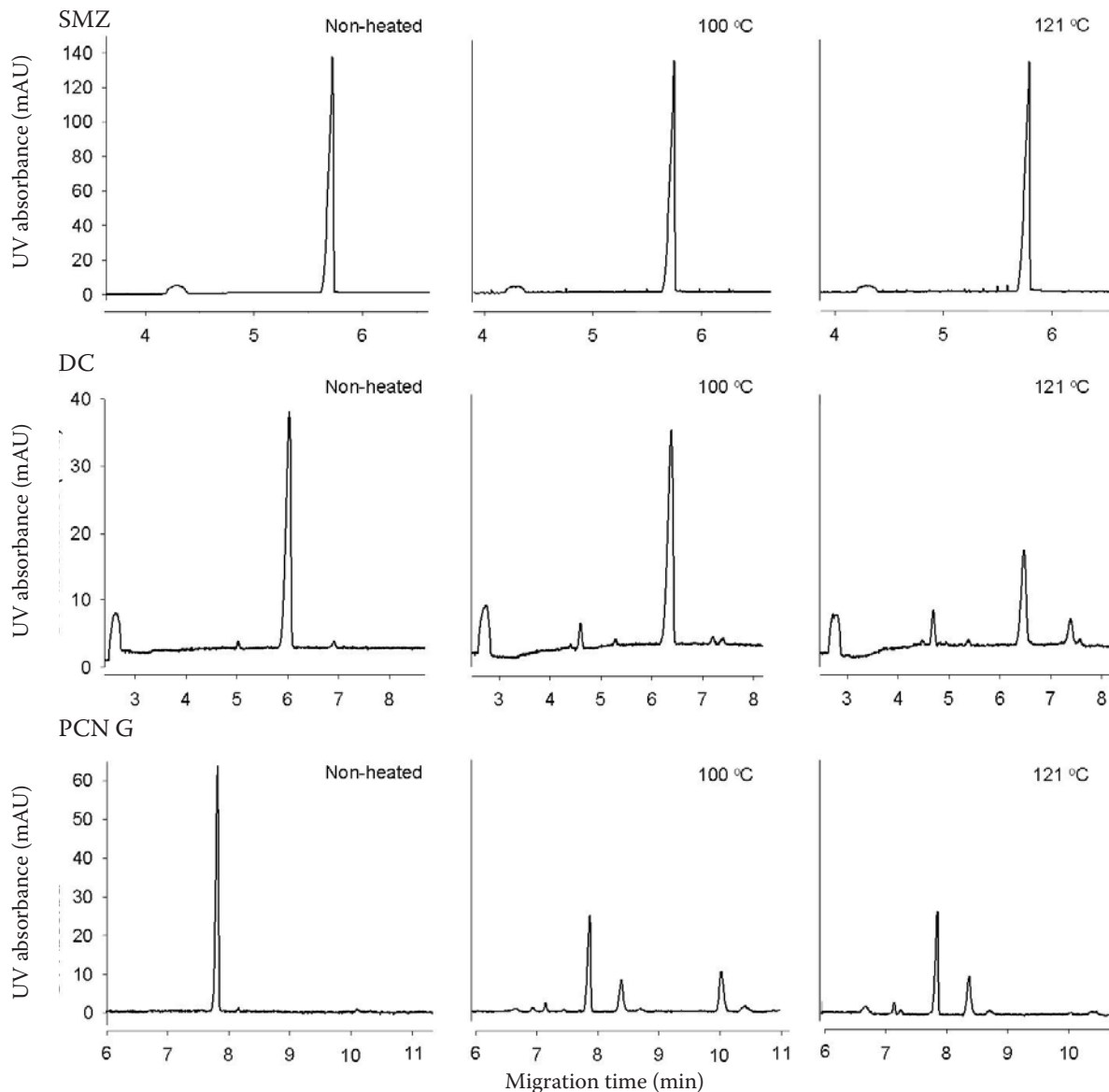

Figure 2. Representative CE electropherograms before and after 15 min heating of SMZ, DC and PCN G in water

Table 1. Quantitative and qualitative changes in electrophoretic peaks of 14 veterinary antibiotics at 200 ppm, after 15 min of heat treatment (n = 3)

Concentration (µg/ml)	100 °C			121 °C		
	number of new peaks	increase of new peak area (%)	total peak area change (%)	number of new peaks	increase of new peak area (%)	total peak area change (%)
Tetracyclines						
CTC	ND	NA	4.4	1	12.5 ± 0.7	64.7
DC	ND	NA	5.9	2	22.3 ± 2.1	77.1
TC	3	48.5 ± 5.2	99.9	ND	NA	76.7
OTC	2	45.7 ± 4.7	120.5	ND	NA	60.5
β-lactams						
DIC	ND	NA	3.7	2	37.3 ± 5.6	87.3
AMP	ND	NA	12.4	3	36.3 ± 2.7	107
OXA	ND	NA	21.4	2	40.8 ± 1.5	107.4
AMX	ND	NA	38.1	3	44.1 ± 1.7	118.9
CLO	1	9.7 ± 1.0	32.6	4	60.2 ± 1.4	127
PCN G	2	56.6 ± 1.0	114.2	1	21.6 ± 0.5	76.9
Sulfonamides						
SMZ	ND	NA	2.1	ND	NA	2.2
SMX	ND	NA	3.1	ND	NA	5.3
Others						
LIN	ND	NA	4.6	ND	NA	6.3
COL	ND	NA	21.8	ND	NA	42.3

NA = denotes not applicable; ND = denotes not detected

of antibiotics at various temperatures were demonstrated by their electropherographic profiles in Figure 2. Sulfamethazine was minimally reduced at both 100 °C and 121 °C showing high heat stability, while DC was stable at 100 °C but degradable at the higher temperature (121 °C). On the other hand, PCN G consistently showed high degradation at both tested temperatures indicating heat lability. The ranking of heat stability by antibiotic classes was only evident at 121 °C sulfonamides were greater than or equal to lincomycin, which was greater than colistin, which in turn was greater than or equal to tetracyclines, which were greater than β-lactams. Therefore, the results reveal differential heat stability patterns of the same class of antibiotics at different temperatures.

In contrast to the main peak decrease in almost all drugs, only certain antibiotics produced quantifiable new peaks after heating (Figure 2, Table 1).

For the purpose of the study, heat-generated new peaks greater than 10% of the main peak area were counted as new peaks. Up to three new peaks were produced for tetracyclines and β-lactams after heating at 100 °C and up to four new peaks were detected for β-lactams at 121 °C with subsequent increases in new peak area and total peak area change (Table 1), further supporting the lower heat stability of these two drug classes. Furthermore, differential degradation profiles were discovered among antibiotics in the same family after heating at 100 °C. For instance, OTC and TC generated two to three new peaks whereas both CTC and DC did not produce any detectable new peaks. Similarly, PCN G was the most heat labile also generating two to three new peaks in contrast to AMX, AMP, DIC and OXA where no new peak were observed. These results suggest that even with drugs that are similar in structure, the degradation pattern can be

different and that this can further vary with heating temperatures. In light of the differential degradation among antibiotics in the same class, we conclude that heat stability based on drug class alone is not reliable, an assertion which is supported by a previous study on amphenicols (Franje et. al., 2010). Sulfonamides, LIN and COL on the other hand were reduced by only about 5%, 12% and 30%, respectively and did not produce any new peak, indicating higher heat stability. However, the increase in peak numbers was not proportional to the increase in new peak area nor did it correlate to the reduction in peak size, suggesting that neither new peak number nor area can be relied on alone for the determination of structural integrity or heat stability. Therefore, although based on the changes in peak number and new peak area, heat stability ranking by drug class was relatively similar to the main peak reduction ranking, which ran sulfonamides equal to LIN equals to COL greater than tetracyclines greater than β-lactams, and the latter parameter (main peak reduction) should be the main determinant. At 200 ppm or lower concentra-

tions of 100 ppm (data not shown) and 50 ppm, the main peak reduction of antibiotics after heating did not differ significantly. This suggests that the effects of heating on the antibiotics were similar at these three concentrations and that decreases in concentration did not cause significant differences. These results support the use of the current approach to estimate the degradation of these drugs at even lower concentrations. Nevertheless, in an actual scenario where the possible level of residues in food may be one ppm or less, whether or not the same behavioural trend could be expected warrants further study.

Changes in the MIC and correlation to structural degradation

Traub and Leonhard (1995) had suggested in a previous study that drugs should be considered heat-labile when MICs are raised more than 16 fold, partially heat-labile when MICs are raised four to eight fold, and heat-stable when MICs are raised

Table 2. Fold increase in minimum inhibitory concentration (MIC) after 15-min heat treatment of 12 antibiotics in water

	Bacillus subtilis		*Staphylococcus aureus*		*Escherichia coli*	
	100 °C	121 °C	100 °C	121 °C	100 °C	121 °C
Tetracyclines						
CTC	1	4	2	16	2	16
DC	1	1[b]	1	2	1	2
OTC	32	256	32	64	32	32
TC	2	128	2	32	2	16
β-lactams						
AMX	2	8	1[a]	4	1[a]	4
AMP	1	2[c]	1	2[c]	1	2[c]
CLO	1[a]	4	2	4	NA	NA
DIC	1	4	1	2	NA	NA
OXA	1	4	1	2	NA	NA
PCN G	32	1024	64	64	NA	NA
Others						
COL	1	1[b]	1	1[b]	1	1[b]
LIN	1	1	1	1	1	1

NA = denotes not available
[a]peak reduction of 30% without MIC change
[b]peak reduction of 50% without MIC change
[c]peak reduction of 70% with only two fold MIC change

less than two fold after autoclaving. In this study, after heat treatments, the MIC increased variably from 2- to 1024-fold for different antibiotics (Table 2) with higher temperatures correlating well with increases in MIC's. Among the 12 individual antibiotics tested, PCN G and the tetracyclines, namely CTC, OTC and TC could be considered largely inactivated by autoclaving while the majority of the β-lactams, namely AMX, CLO, DIC and OXA were determined to be partially heat-labile. Doxycycline, AMP, COL and LIN were found to be heat stable. Studies on the heat stability of tetracyclines in food performed as early as 1959 with the use of microbiological methods (O'Brien et al., 1981) and more recently in HPLC studies (Rose et al., 1996; Gratacos-Cubarsi et al., 2007) all indicate that tetracyclines are not very resistant to heat. In this study, TC and OTC, together with PCN G were shown to be highly heat-labile with peak reductions as high as 99% and MIC increases from 16- up to 1024-fold after autoclaving. In contrast, LIN and COL were minimally reduced and showed

no changes in their MIC after autoclaving, confirming their high heat stabilities. However, it should be noted that at 100 °C, most drugs except for OTC and PCN G had an equal or less than two-fold increase in their MIC, suggesting that activity-wise these drugs are quite stable in response to short-time (15 min) boiling. The ranking of heat stability based on MIC results was: LIN, COL equal to DC, AMP and greater than or equal to CLO, DIC, OXA, and greater than AMX, and greater than CTC, and greater than TC, OTC, PCN G.

Further analysis correlating the degree of main peak reduction and the fold increase in MIC indicated that structural degradations generally correlated well with the increase in MIC (i.e., the decrease of antimicrobial activity). However, the degree of reduction was not always proportional to the fold increase in MIC (Table 2). For instance, despite 70% structural degradation of AMP after autoclaving, its MIC had increased only two-fold while a 50% degradation of DC resulted in no change in MIC (against *B. subtilis*). A complete correlation

Table 3. Ames/mutagenicity test showing revertant changes induced by heated CTC (at 100 °C for 15 min)

Compound	Concentration (µg/plate)	Number of revertants (colony/plate)			
		Salmonella TA 98		*Salmonella* TA 100	
		−S9	+S9	−S9	+S9
NC		11.0 ± 10.8	27.3 ± 3.7	10.7 ± 1.3	12.0 ± 1.4
PC		139.0 ± 13.5*	1644.0 ± 374.1*	2053.3 ± 302.3*	225.7 ± 21.9*
Chlortetracycline					
	0.0625	7.7 ± 2.5	19.0 ± 6.7	15.7 ± 4.5	16.0 ± 2.5
	0.125	14.7 ± 13.8	24.3 ± 0.9	16.3 ± 4.5	12.3 ± 2.6
Non heated	0.25	11.0 ± 7.3	25.0 ± 2.8	19.3 ± 3.1	9.3 ± 2.6
	0.5	22.0 ± 4.6	23.3 ± 2.9	13.3 ± 2.4	14.0 ± 2.5
	1	25.7 ± 8.2	25.7 ± 2.1	17.0 ± 1.4	12.0 ± 1.6
NC		19.7 ± 2.5	30.3 ± 4.0	256.0 ± 17.1	7.7 ± 1.9
PC		257.7 ± 8.3*	3170.7 ± 249.4*	2579.3 ± 130.6*	154.0 ± 14.4*
Chlortetracycline					
	0.25	17.3 ± 3.4	27.3 ± 2.1	274.7 ± 14.4	12.7 ± 1.3
	0.5	19.7 ± 0.5	32.0 ± 2.2	247.0 ± 1.6	13.3 ± 4.2
Heated	1	20.7 ± 0.9	28.7 ± 2.9	267.3 ± 34.1	7.7 ± 1.7
	2	10.3 ± 1.9	44.3 ± 6.7	213.3 ± 30.7	12.7 ± 4.1
	4	15.0 ± 2.2	73.0 ± 3.7*	212.0 ± 37.0	42.7 ± 4.5*

NC and PC = normal and positive control
*indicates > two fold change compared to NC

Bacillus subtilis

Staphylococcus aureus
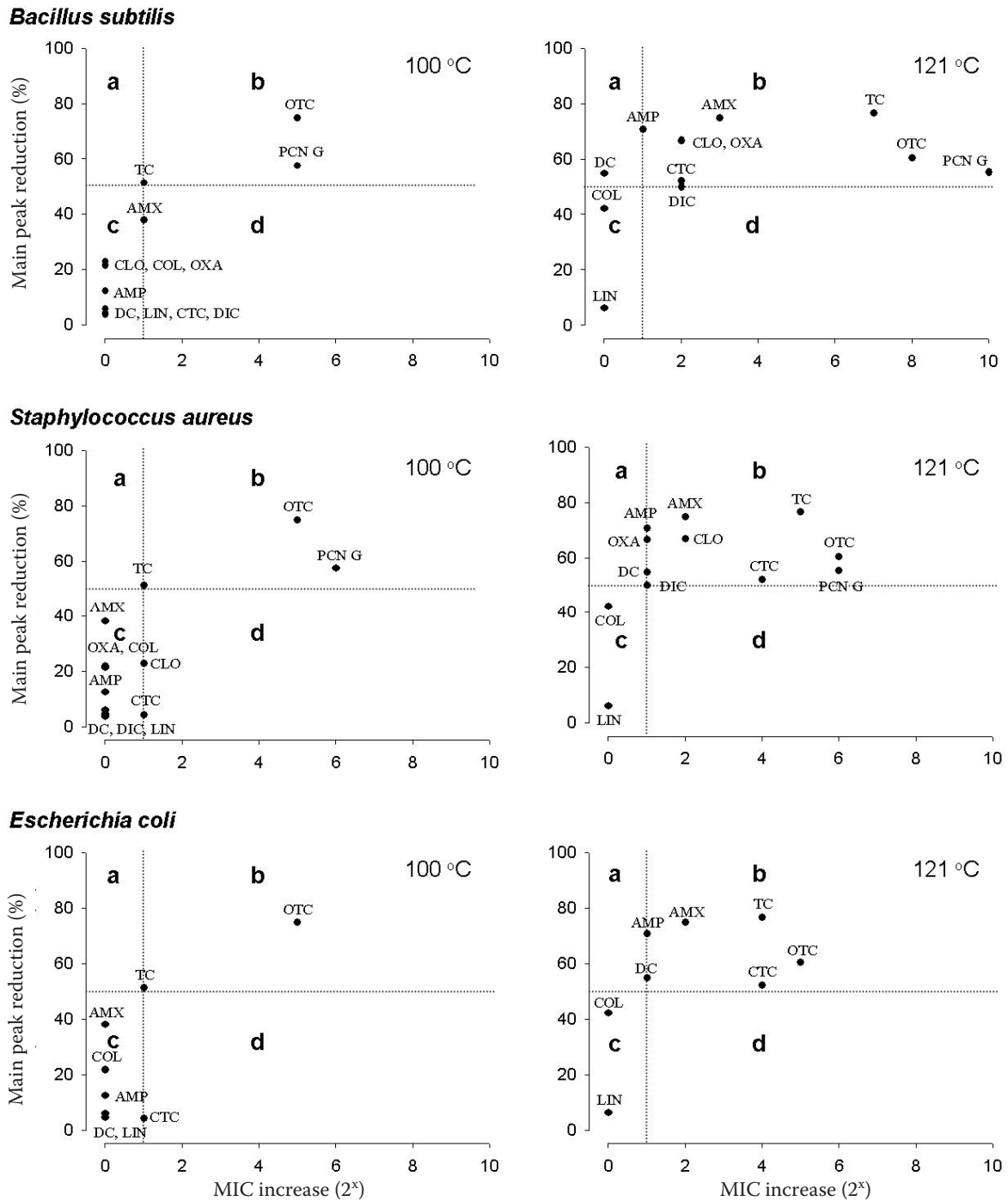

Escherichia coli

Figure 3. Correlation between main peak reduction (MPR) and MIC increase in tested bacteria after boiling or autoclaving of antibiotics in water; > 50% MPR with ≤ two fold MIC increase (**a**), > 50% MPR with > two fold MIC increase (**b**), < 50% MPR with ≤ two fold MIC increase (**c**), and < 50% MPR with > two fold MIC increase (**d**)

of the main peak reduction with the MIC increase after heating is shown in Figure 3. It is noteworthy that some antibiotics, in spite of more than a 50% reduction were still antimicrobially active with only lesser than or equal to two-fold MIC increases. The effects were more apparent after autoclaving (121 °C), which showed that AMP and DC could

actively inhibit the growth of the three tested bacteria in spite of being structurally degraded (Figure 3 right panels). Feasible explanations for this inconsistency include possible antimicrobial activity from the degradation products. On the other hand, the majority of tetracyclines and β-lactams were reduced by more than 50% with significantly

raised MICs (4- to 1024-fold) after 121 °C heating (Figure 3 upper right compartment, b), which was in line with their structure-activity relationship. Lincomycin and COL were highly heat stable showing less than 50% reduction and less than two-fold MIC increases (Figure 3 lower left compartment, c). As expected, no drugs were seen to be reduced by less than 50% and to incur a greater than two-fold MIC increase (Figure 3 lower right compartment, d). While identification of the degradation structures was not the main purpose of the current study, these results highlight the possibility that residual antibiotics may be structurally degraded by heat treatment without a significant decrease in their antimicrobial activity. The UV-PDA spectra of the generated new peaks (data not shown), exhibited insignificant changes in spectra from the original main peak, suggesting that the new degradation products probably maintained a very similar structure to the parent drug. These findings suggest that degradation products of antibiotic residues resulting from heat treatments might still contain various degrees of antimicrobial activity (Franje et al., 2010), and hence cannot always be assumed safe.

Mutagenicity (Ames) test

The production of many new peaks without a parallel diminishment of antimicrobial ability prompted us to further investigate any harmful activities that might be induced by these newly-formed compounds, including genotoxic effects of the heat-treated antibiotics. Selected antibiotics including CTC, DC, AMX and DIC were tested for mutagenicity after heating. To the best of our knowledge, few studies had reported on heat-degraded products and their ability to cause genotoxicity or mutagenicity. The Ames test evaluates the mutagenic potential of chemicals by studying their effects on one or more histidine-requiring strains of *Salmonella typhymurium* in the absence and presence of a liver metabolizing system (S9 microsome). Salmonella strains TA98 and TA100 are sensitive strains that are commonly used for detection of specific frame-shift point mutations and base pair mutations respectively (Lolo et al., 2006). A compound was considered a mutagen if the tested sample produced a response, which was at least twice as high as the one found with the negative control (Maron and Ames, 1983) and a dose-

effect relationship was observed. Table 3 shows that heated CTC demonstrated a dose-dependent increase in histidine revertants in the presence of hepatic enzyme (S9). At 4 μg/plate of autoclaved CTC, a two-fold increase in His+ revertants over the negative control in Salmonella TA98 and six-fold doubling in TA100 were observed, suggesting the mutagenicity of heated CTC and hinting that the metabolites of heat-degraded CTC may possess mutagenicity. Doxycycline, AMX and DIC on the other hand, although being degraded by up to 75%, did not show significant revertant activity (data not shown). TC and OTC, due to their total inactivation both structurally and microbiologically, were not tested for the Ames assay. This possible unfavorable transformation of structure by heating is relevant to public food safety in that consumers might be exposed not only to residual antibiotics but also to their possibly toxic degradation products generated after heating. While the long term effects of broken-down antibiotic residues remain to be studied, the possibility of harmful effects arising from previous exposure to antibiotics and its thermobreakdown products should not be ignored. The present findings also suggest that it is unsafe to rely solely on heating to destroy antibiotic residues due to variable degradation after heating and the formation of possibly toxic breakdown products. To ensure that no residues would be in food, strict observance of the proper withdrawal period for each drug administered to domestic animals is imperative. Although heating in water did not reflect all cooking conditions, the results from simulated heating in liquid media could provide valuable information in interpreting results from certain actual cooking conditions (Moats, 1988). Moreover, heating in water avoids the lengthy lag phase of heating commonly encountered in the cooking of solid tissue and can thus easily assess the actual exposures of antibiotics to heat (Hassani et al., 2008).

In conclusion, antibiotics vary in their susceptibility to degradation by heating processes. With the utilization of both CE and MIC assays for evaluation of structural degradation and changes in antimicrobial activity, differential heat stability of antibiotics after heating was conclusively demonstrated. Although structurally degraded and with accompanying increases in MIC's (to different degrees), the heat treatment of some antibiotics at above 100 °C may generate degradation products that contain certain mutagenic properties as

demonstrated by the Ames test. Therefore, toxicity can become unpredictable and the degradation of antibiotics cannot always be assumed to render food safe for consumption. These data improve our understanding of the effects of boiling treatments on multiple antibiotic classes that might reach consumers if safety precautions are not followed.

REFERENCES

Anadon A, Martinez-Larranaga MR (1999): Residues of antimicrobial drugs and feed additives in animal products: regulatory aspects. Livestock Production Science 59, 183–198.

Arriaga-Alba M, Barron-Moreno F, Flores-Paz R, Garcia-Jimenez E, Rivera-Sanchez R (1998): Genotoxic evaluation of norfloxacin and pipemidic acid with the Escherichia coli Pol A–/Pol A+ and the ames test. Archives of Medical Research 29, 235–240.

Chlopkiewicz B, Ejchart A, Marczewska J, Anuszewska E, Priebe W (2005): Evaluation of mutagenic and genotoxic activities of new derivatives of anthracyclines. Acta Poloniae Pharmaceutica 62, 99–104.

CLSI (Clinical Laboratory Standards Institute) (2008): Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically M7-A7. CLSI, Wayne, PA, USA.

Franje CA, Chang SK, Shyu CL, Davis JL, Lee YW, Lee RJ, Chang CC, Chou CC (2010): Differential heat stability of amphenicols characterized by structural degradation, mass spectrometry and antimicrobial activity. Journal of Pharmaceutical and Biomedical Analysis 53, 869–877.

Fuh MR, Chu SY (2003): Quantitative determination of sulfonamide in meat by solid-phase extraction and capillary electrophoresis. Analytica Chimica Acta 499, 215–221.

Gocke E (1991): Mechanism of quinolone mutagenicity in bacteria. Mutation Research 248, 135–143.

Gratacos-Cubarsi M, Garcia AF, Picouet P, Pamplona AV, Regueiro JAG, Castellari M (2007): Formation of tetracycline degradation products in chicken and pig meat under different thermal processing conditions. Journal of Agricultural and Food Chemistry 55, 4610–4616.

Hassani M, Lazaro R, Perez C, Condon S, Pagan R (2008): Thermostability of oxytetracycline, tetracyclines, and doxycycline at ultrahigh temperatures. Journal of Agricultural and Food Chemistry 56, 2676–2680.

Hows MEP, Perret D, Kay J (1997): Optimisation of a simultaneous separation of sulphonamides, dihydrofolate reductase inhibitors and ß-lactam antibiotics by capillary electrophoresis. Journal of Chromatography A 768, 97–104.

Ibrahim A, Moats WA (1994): Effect of cooking procedures on oxytetracycline residues in lab muscle. Journal of Agricultural and Food Chemistry 42, 2561–2563.

Isidori M, Lavorgna M, Nardelli A (2005): Toxic and genotoxic evaluation of six antibiotics on non-target organisms. Science of the Total Environment 346, 87–98.

Kang JW, Vankeirsbilck T, Van Schepdael A, Orwa J, Roets E, Hoogmartens J (2000): Analysis of colistin sulfate by capillary zone electrophoresis with cyclodextrins as additives. Electrophoresis 21, 3199–3204.

Kitts DD, Yu CW, Burt RG, McErlane K (1992): Oxytetracycline degradation in thermally processed. Journal of Agricultural and Food Chemistry 140, 1977–1981.

Kuhne M, Hamscher G., Korner U, Schedl D, Wenzel S (2001a): Formation of anhydrotetracycline during a high-temperature treatment of animal-derived feed contaminated with tetracycline. Food Chemistry 75, 423–429.

Kuhne M, Korner U, Wenzel S (2001b): Tetracycline residues in meat and bone meals. Part 2: the effect of heat treatments on bound tetracycline residues. Food Additives and Contaminants 18, 593–600.

Lolo M, Pedreira S, Miranda JM, Vazquez BI, Franco CM, Cepeda A, Fente C (2006): Effect of cooking on enrofloxacin residues in chicken tissue. Food Additives and Contaminants 23, 988–993.

Mamani MCV, Farfan JA, Reyes FGR, Rath S (2006): Simultaneous determination of tetracyclines in pharmaceuticals by CZE using experimental design. Talanta 70, 236–243.

Maron DM, Ames BN (1983): Revised methods for the Salmonella mutagenicity test. Mutation Research 113, 173–215.

McCann J, Choi E, Yamasaki E, Ames B (1975): Detection of carcinogens as mutagens in the Salmonella/microsome test: Assay of 300 chemicals. Proceedings in National Academy of Science USA 72, 5135–5139.

Moats WA (1999): The effect of processing on veterinary residues in foods. Advances in Experimental Medicine and Biology 459, 233–241.

Mortelmans K, Zeiger E (2000): The ames Salmonella/microsome mutagenicity assay. Mutation Research 455, 29–60.

Mourot D, Loussourorn S (1981): Sensitivity of lactic acid bacteria to antibiotics used in veterinary medicine (in French). Recueil de Medecine Veterinaire 157, 175–177.

O'Brien JJ, Campbell N, Conaghan T (1981): Effect of cooking and cold storage on biologically active antibiotic residues in meat. Journal of Hygiene 87, 511–523.

Papapanagiotou EP, Fletouris DJ, Psomas EI (2005): Effect of various heat treatments and cold storage on sulphamethazine residues stability in incurred piglet muscle and cow milk samples. Analytica Chimica Acta 529, 305–309.

Podhorniak LV, Leake S, Schenck FJ (1999): Stability of tetracycline antibiotics in raw milk under laboratory storage conditions. Journal of Food Protection 62, 547–548.

Rose MD, Shearer G, Farrington WHH (1995): The effect of cooking on veterinary drug residues in food: 3. Sulfamethazine. Food Additives and Contaminants 12, 739–750.

Rose MD, Bygrave J, Farrington WHH, Shearer G (1996): The effect of cooking on veterinary drug residues in food: 4. Oxytetracycline. Food Additives and Contaminants 13, 275–286.

Traub WH, Leonhard B (1995): Heat stability of the antimicrobial activity of sixty-two antimicrobial agents. Journal of Antimicrobial Chemotherapy 35, 149–154.

van Egmond HJ, Nouws JFM, Schilt R, van Lankveld-Driessen WDM, Streutjens-van Neer EPM, Simons FGH (2000): Stability of antibiotics in meat during a simulated high temperature destruction process. EuroResidue IV 29, 430–437.

Corresponding Author:

Chi-Chung Chou, National Chung-Hsing University, College of Veterinary Medicine, Department of Veterinary Medicine, 250-1 Kuo-Kuang Rd, Taichung 402, Taiwan

Tel. +886 4 22840405, Fax +886 4 22862073, E-mail: ccchou@nchu.edu.tw