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Identification of *Klebsiella pneumoniae* in microwavable chicken curries using 16s rRNA analysis

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Abstract

Microwave heating technology is widely used in food catering and domestic households. Non-uniformity of microwave temperature distribution causes the formation of hot and cold spots in heated product has led to the survival of foodborne pathogens which may later cause food borne diseases. It is the aim of this study to determine the effect of different microwave heating on the microbiological quality of microwavable frozen chicken curries. Nine commercialised frozen chicken curries were thawed overnight in the chiller (< 4°C) and exposed to different microwave power levels and times (270 W at 60 s; 950 W at 150 s and 300 s). 25 g of chicken curry were homogenised, serial diluted and enumerated aseptically. Total Aerobic Count (TAC), Total Coliform Count (TCC), *Escherichia coli* count and *Salmonella* count were carried out and no microorganisms were detected. Enterobacteriaceae were found in the food samples after enrichment process. Polymerase Chain Reaction (PCR) amplification and 16S ribosomal ribonucleic acid (rRNA) sequencing were carried out. Results of 16S rRNA sequence analysis indicated that two gram-negative isolates after enrichment were identified as *Klebsiella pneumoniae.*

Introduction

Microwave is widely used in food processing and domestic households as it is convenient for heating and serving food. Microwave heating uses electromagnetic waves at definite frequencies to produce heat energy in food materials and this energy are more dependable for pasteurisation and sterilisation purpose compared to conventional heating way as microwave heating can achieve the desired temperature in a more rapid way within shorter time period (FDA, 2012). Microwave heating involves heating the food in the packaging material (wrapping, film, container) in the microwave oven (Nerín *et al*., 2003). However, microwave heating can caused uneven heat distribution that led to temperature differences in the heated products (Vadivambal and Jayas, 2007). Several studies showed that microwave heating were able to reduce or inactivate foodborne pathogens including *Camplylobacter jejuni* (Dąbrowski *et al*., 2009), pathogenic *E. coli* O157:H7 (Apostolou *et al*., 2005; Picouet *et al*., 2009), *Listeria monocytogenes* (Benlloch-Tinoco *et al*., 2014), *Staphylococcus aureus* (Gedikli *et al*., 2008) and *Salmonella* (Anaya *et al*., 2008) in different food products. On the other hand, non-uniform heating in microwave led to the survival of food borne pathogens including *E. coli* K12 and *C. jejuni* in specific cold spots in heated food associated with insufficient microwave heating treatment time (Göksoy *et al*., 2000).

Klebsiella are members of the normal intestinal flora of humans and animals and are also found in the normal flora of skin and mouth (Puspanadan *et al*., 2012). Most of the nosocomial *Klebsiella* infections are caused by *K. pneumoniae* and medically, it is categorised as the most important species of the genus (Gautam *et al*., 2015). Consumption of contaminated fish (Haryani *et al*., 2007) and water (Podschun and Ullmann, 1998) had been linked to infections by *Klebsiella*. In 2008, a hospital outbreak of extended-spectrum β-lactamase (ESBL)-producing *K. pneumoniae* was detected. 35% of the hospital surfaces or foodstuffs were colonised while 14% of the 44 food handlers were found to be faecal carriers (Calbo *et al*., 2011) while an earlier food poisoning case isolated *K. pneumoniae* from a contaminated hamburger (Sabota *et al*., 1988). In Malaysia, Haryani *et al.* (2007) reported the first occurrence of K. pneumoniae in street foods. Food products had been identified as a potential transmission vector for *K. pneumoniae.* However, there is very little published information on microwavable foods. Hence, this study aims to investigate the microbiological quality of microwavable frozen chicken curries at different

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cooked stages and the presence of cold spots within the microwavable food.

Materials and Methods

Equipment and samples

Nine frozen microwavable chicken curries were purchased and all samples were transported in an insulated ice box containing ice (<4°C) to the laboratory within 2 hours of purchase (Aziz *et al*., 2002; Apostolou *et al.*, 2005). The samples were allowed to thaw (<4°C) in the chiller overnight before microwaving. The samples were packaged in polyphenylene ether (PPE) materials and came with a self-venting design hence no puncture was required prior to microwaving. Domestic microwave oven model NN GD371M-Panasonic (frequency 2450 MHz, output power 950 W, Panasonic Corporation, Osaka-Japan) was used at different power levels (270 Watts for 60 seconds (s) and 950 W for 150s and 300s) to microwave the chicken curry. The different powers were used to simulate the conditions of low, medium and high temperature of microwaving. Internal temperature was measured using digital thermometer (ETP 109, All sun) at 5 different points (lower left, upper left, centre, upper right and bottom right) in the microwavable chicken curries (Figure 1). Each determined spot was measured at random and the temperature recorded.

Microbiological analysis of frozen chicken curries

A total of 25 g chicken curries were weighed and homogenised with 225 ml of sterile 0.2% buffered peptone water (BPW, Merck KGaA, Darmstadt, Germany) for 2 minutes. Serial dilution up to 10-5 was carried out and 1ml aliquot from each dilution was triplicate plated on sterile plate count agar (PCA) and violet red bile lactose (VRBL) agar (Friedemann Schmidt, Australia). The plates were incubated at 37°C for 24 hours. Another 1ml aliquot was transferred to Lauryl Sulphate Tryptose (LST) broth (Merck, Germany) for presumptive test for coliform bacteria. An enrichment process was carried out with 0.2% buffered peptone water (BPW, Merck KGaA, Darmstadt, Germany) followed by enrichment in Enterobacteriaceae enrichment (EE) broth which was then streaked on violet red bile lactose (VRBL) agar (Friedemann Schmidt, Australia). The plates were incubated at 37°C for 24 hours.

Enumeration of *E. coli* was carried out with a different 25 g of sample and homogenised with sterile Brain Heat Infusion (BHI) broth (Merck, Germany). BHI broths were incubated at 37°C for the first 3 hours and then transferred to 225 ml double strength tryptone phosphate broth (Merck, Germany) and incubated for another 21 hours at 37°C. A loop-full of cultured medium was streaked on sterile Levine's Eosin-Methylene Blue (L-EMB) agar and MacConkey agar (Merck, Germany) and incubated for 20 hours at 37°C.

Enumeration of *Salmonella* was carried out with an extra 25 g of samples and homogenised with sterile lactose broth (Merck, Germany). pH of lactose broth was adjusted after incubation of 1 hour at room temperature with the cap tightened and the cultured medium was then incubated for 24 hours at 37°C with the cap loosened. A total of 1ml of cultured medium was then transferred to Selenite Cystine (SC) broth(Sigma-Aldrich, USA) and Rappaport-Vassiliadis (RV) medium (Merck, Germany) and incubated for 24 hours at 37°C. After incubation, one loopful of aliquot from SC broth and RV medium were streaked on Xylose Lysine Deoxycholate (XLD) agar (Merck, Germany) and incubated for 24 hours at 37°C (Harrigan, 2007).

Triple Sugar Iron (TSI) agar and Simmon's citrate agar (Sigma-Aldrich, USA) were used to differentiate between Enterobacteriaceae. Kovac's Indole reagent (Merck, Germany) was used for indole test to distinguish between indole-positive and indole-negative microorganisms. Methyl-red and Voges-Proskauer (MR-VP) test was carried differentiate between *E. coli* and *Klebsiella* sp. (Merck, 2005; Harrigan, 2007).

DNA extraction and agarose gel electrophoresis

Enterobacteriaceae samples were preserved using 15% glycerol stock. Bacteria cells were then inoculated into 5 ml Luria Bertani broth (Merck, Germany) and incubated overnight until the optical density (OD) value of culture falls within the range of 0.8-1.0 at 600 nm (Kinge *et al*., 2008; Magray *et al*., 2011). Genomic DNA were then extracted (Fattahi *et al.*, 2013) using G-spin Total Genomic DNA Kit (Intron, Korea). Horizontal agarose 1% w/v slab gel was prepared using agarose (Vivantis, USA), 1X Tris-Acetate-EDTA (40 mMtris-acetate; 20 mM glacial acetic acid and 1.0 mM EDTA, pH 8.0) and 10 µl of 1 mg/ml ethidium bromide (Kinge *et al*., 2008). DNA ladder of 1 kb and 100 bp and each DNA template with loading dye (Vivantis, USA) were loaded into the well prior to electrophoresis. The agarose gel was electrophoresed for 45 minutes at 80 V and EtBr-stained agarose gel was visualised under UV transilluminator (Kazemi and Hajizadeh, 2012).

Polymerase Chain Reaction (PCR)

Extracted DNA from isolated bacteria were

Table 1. Average internal temperature of microwaved chicken curries at 270 W and 950 W

Microwave power (W)	Treatment Time (s)	Average internal temperature (°C)					Range (max - min temperature [°C])
		UL	LL	UR	LR	C	
270	60	-2.56±0.71	-2.53±1.29	-2.21±0.68	-2.59±0.56*	-1.79±0.31**	0.80
950	150	17.94±6.29	22.04±4.49**	4.86±2.88*	15.14±5.41	18.28±5.63	17.19
	300	55.97±3.96	53.39±3.62	63.67±3.88**	50.70±6.56*	55.19±5.78	12.97

** Max. value; * Min. value; UL= upper left; LL= lower left; UR= upper right; LR= lower right; C=centre

then used for PCR and 16S rRNA analysis using 16S rRNA primers 27F (Forward primer 5'- AGAGTTTGATCCTGGCTCAG -3') and 1492R (Reverse primer 5'- GGTTACCTTGTTACGACTT-3') (Paju *et al.*, 2003; Magray *et al*., 2011). PCR reaction was carried out in 50 µl PCR reaction mixture (2.5 U Taq DNA polymerase, 10 p moles each of the forward and reverse primers, 1X Taq buffer, 0.2 mM dNTP mix, 1.5 mM $MgCl_2$, DNA template and sterile distilled water) (1st Base, Singapore). PCR amplification was performed in a Thermal Cycler (Eppendorf AG 22331 Hamburg, Germany) under conditions such as heat denaturation at 94°C for 4 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute; annealing at 59°C for 1 minute and extension at 72°C for 1 minute. This was followed by a final extension step at 72°C for 7 minutes and 4°C hold (Kinge *et al*., 2008; Pathak *et al.*, 2013).

16S rRNA sequencing

PCR products were subjected to 16S rRNA fragment sequencing. The relative molecular sizes of the PCR products were compared against the sequences from the Gen Bank database, National Centre for Biotechnology Information (NCBI) using Basic Local Alignment Search Tool (BLAST) analysis program (Fattahi *et al*., 2013). Sequences obtained were checked (Sequence Scanner V1.0, Thermo Fisher Scientific), cleaned (Hall, 1999; Larkin *et al.*, 2007) and a maximum likelihood phylogenetic tree was ran to show the percentage of similarity (Felsenstein, 1985; Kimura, 1980; Kumar *et al*., 2016).

Results and Discussion

Bacterial growths were found in all samples after enrichment. There was no bacterial growth observed in Aerobic Plate Count and Total Coliform Count. Internal temperature of food sample was recorded after microwave treatment at different microwave power levels and times in order to determine the presence of hot and cold spots in the food samples. The average internal temperature achieved was 55.78±4.85°C after being treated at 950 W for 300 s. Large temperature deviations were observed at different points (right upper and lower, left upper and lower, centre) of the food samples as tabulated in Table 1 and Figure 1. There exist hot and cold spots within the food samples after microwave treatment indicating that uneven temperature distribution occurred in the food even after microwave treatment for 5 minutes. The highest temperature achieved was 63.67±3.88°C (upper right region) which was not sufficient to inhibit bacteria growth. According to Apostolou *et al.* (2005) foodborne pathogen such as *E. coli* O157:H7 requires an endpoint temperature of 73.7°C.

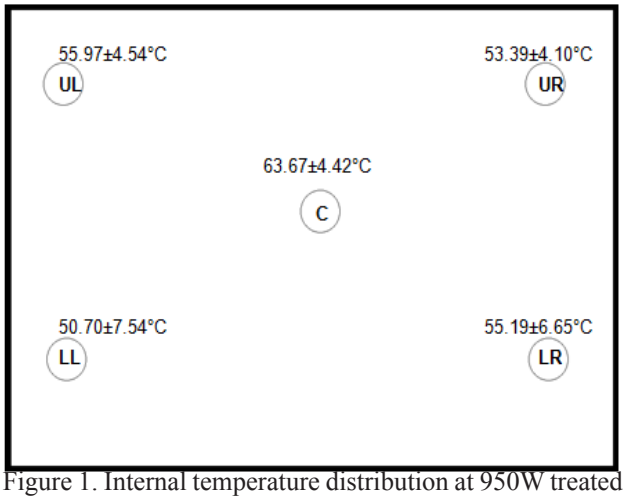


Figure 1. Internal temperature distribution at 950W treated for 300 s
UL= upper left; LL= lower left; UR= upper right; LR= lower right; C=centre

Enterobacteriaceae were only revived and detected after enrichment as viable cells may be injured during microwave heating. Bacterial growth was observed in SC broth (Sigma-Aldrich, USA) and RV medium (Merck, Germany) but there was no typical *Salmonella* colonies found in the food samples. Typical *Salmonella* colonies will produce

Table 2. Biochemical test on bacteria enumerated from sample S8 and R8

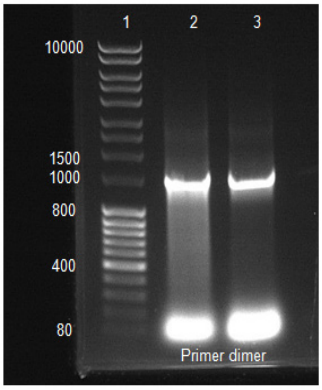
Sample	Biochemical test							
	GS	Shape	TSI	SCA	Indole	MR	VP	SIM
R8	-	rod	K/A/G	+	-	-	+	-
S8	-	rod	A/A/G	+	-	-	+	-

*- = Negative;
*+ = Positive;
*K/A/G= Glucose fermentation; Peptone catabolisation; Gas producing
*A/A/G= Glucose/Lactose/Sucrose fermentation ; Gas producing
*R= colonies from RV
*S= colonies from SC
*GS= Gram's staining

hydrogen sulphide and turn the colonies into black colour but the agar remained orange and yellow mucoid colonies were detected from two samples (R8 and S8). Biochemical tests were carried out for samples R8 and S8 and the results were tabulated in Table 2 and verified according to Breed *et al.* (1957), where *Klebsiella pneumoniae* is rod-shaped, Indole-test and Methyl red test negative, usually produces acetylmethylcarbinol (negative VP test) and utilise ammonium citrate as source of carbon.

Genomic DNA extraction of two strains of bacteria enumerated from sample R8 and S8 was carried out followed by PCR (Figure 2). PCR products were sent to MyTACG Bioscience Enterprise without purification and sequenced using Sanger method. The sequencing results were obtained from MyTACG Bioscience Enterprise and the sequences were checked and cleaned. The two isolates were aligned using nucleotide BLAST to ascertain the sequence similarity against the NCBI database. Based on the results obtained, the genera with similar identity was *Klebsiella* in two of the microwavable chicken curries. A molecular phylogenetic analysis by maximum likelihood method was carried out with *Klebsiella pneumoniae* and *Shigella sonnei* and *Enterobacillus tribolii* as outgroup. It showed that the sequences obtained from samples R8 and S8 were grouped to *Klebsiella* sp. or *Klebsiella pneumoniae* at 99% likelihood (Figure 3).

Klebsiella pneumoniae is able to cause pneumonia or destructive lung inflammation diseaseand progressed to bacteraemia, followed by endocarditis, liver abscess and other systemic septic complications (Puspanadan *et al.*, 2012; Hwang *et al.*, 2013). In previous studies, *Klebsiella pneumoniae* was successfully isolated from different food samples including raw meat (Gwida *et al.*, 2014; Prasad Sharma and Chattopadhyay, 2015), chicken eggs (Sabarinath and Guillaume, 2012), raw vegetables (Puspanadan *et al.*, 2012), ready-



Lane 1: ThermoFisherMassRuler Mix
Lane 2: Sample R8 PCR product
Lane 3: Sample S8 PCR product

Figure 2. Quantification of PCR products on 1% agarose gel

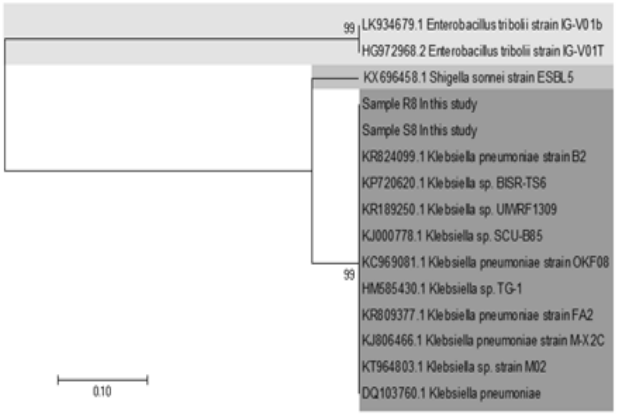


Figure 3. Molecular phylogenetic analysis by Maximum Likelihood method of samples R8 and S8

to-eat vegetables (Kim *et al.*, 2015), fish products (Diana and Manjulatha, 2012), street vended foods (Tambekar *et al.*, 2011; Feglo and Sakyi, 2012) and powdered infant formula (Liu *et al.*, 2008; Zhou *et al.*, 2011). In recent years, extended-spectrum β-lactamase (ESBL)-producing *Klebsiella* had been isolated from chicken meat (Overdevest *et al.*, 2014) chicken broilers (Wu *et al.*, 2016).

Enterobacteriaceae are not particularly heat resistant as normal pasteurisation and cooking processes used by food industry will inactivate these bacteria (Lawley *et al.*, 2012). Although *K. pneumoniae* found in food products are not always pathogenic, however *K. pneumoniae* is an opportunistic pathogen and primarily affects immune-compromised individuals (Podschun and Ullmann, 1998). These foodborne opportunistic pathogen may contaminate chicken products from raw poultry meat and if uncooked appropriately, these bacteria may subsequently cause illnesses in humans who consume the contaminated food (Smet *et al.*, 2010; Wu *et al.*, 2016).

Conclusion

Microwave heating treatments show potential effects at certain frequency or power levels and heating times in inhibiting microorganisms or foodborne pathogens. After microwave heat treatments, no aerobic microorganisms, coliform, *E. coli* or *Salmonella* were detected. However, after the enrichment process, two strains of *Klebsiella pneumoniae* were detected in two microwaved chicken curry samples. It is probable that during microwave heating, an uneven heat distribution in the working cavity was created and the presence of cold spots led to the survival of *Klebsiella pneumoniae*. Chicken products may serve as a potential route in which *Klebsiella* is transmitted to humans – particularly among immune-compromised individuals and subsequently result in illnesses.

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