UNIVERSITI PUTRA MALAYSIA

SURVEILLANCE OF VIBRIO PARAHAEMOLYTICUS IN SHELLFISH PURCHASED FROM SELECTED RETAILERS IN SELANGOR, MALAYSIA

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By

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Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Master of Science

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

SURVEILLANCE OF *VIBRIO PARAHAEMOLYTICUS* IN SHELLFISH PURCHASED FROM SELECTED RETAILERS IN SELANGOR, MALAYSIA

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September 2015

Chairman: Professor Son Radu, PhD
Faculty: Food Science and Technology

The aims of this study were to optimize the multiplex polymerase chain reaction (mPCR) assay for the detection of *V. parahaemolyticus* and the respective pathogenic strains (*tdh+* and/or *trh+*), and to compare with the loop-mediated isothermal amplification (LAMP) assay. These assays were not limited to detection only, and likewise coupled with the most probable number (MPN) to quantitate the bacterium in various shellfish samples obtained from wet markets and hypermarkets. The surveillance data were later adopted into a stochastic microbial risk assessment (MRA) model to evaluate the public health risk. In addition to these, a kitchen simulation was conducted to provide cross-contamination and decontamination data.

A total of 232 samples comprising of bloody clams, surf clams and shrimps were randomly purchased from the wet markets and hypermarkets in Selangor. 229 (98.7%) of the samples were positive for *V. parahaemolyticus* with counts ranging from 30 to >110, 000 MPN/g. Positive samples for *tdh+ V. parahaemolyticus* were obtained in 77 out of 232 (33.1%) samples ranging from 30 to >110, 000 MPN/g. Meanwhile, positive samples for *trh+* were identified in 16 out of 232 (6.9%) samples examined ranging from 30 to 9,600 MPN/g. In addition, we found that LAMP was rather more robust and sensitive compared to the multiplex PCR.

Bloody clam was selectively picked for a thorough MRA as the pathogenic strains of *V. parahaemolyticus* were prevalent in this sample. The study framework was established based on the currently available experimental and survey data in combination with the @RISK software to simulate the uncertainties based on the Monte Carlo simulation. The estimated risk was valued as 1.04E-4/daily serving/person. This translates to an estimate of 250 to 197,000 cases yearly.

The simulation of the handling of bloody clams in domestic kitchens was designed to imitate real events in domestic kitchens as much as possible to give a realistic quantitative data on how *V. parahaemolyticus* could cross-contaminate to other ready-to-eat (RTE) foods (eg, lettuce). It was found that 20.40 ±13.78% of the total *V. parahaemolyticus* population from the bloody clams were transferred to the hands and kitchen utensils through primary cross-contamination, and the cut lettuce had an average of 7432.5 MPN/g of *V. parahaemolyticus*. The attempted cleanings reduced the transferred population by 97.63 ± 3.43% (water) and 96.00 ± 5.03% (cloth), and the cut

lettuce had an average of 9.27 MPN/g of *V. parahaemolyticus*. Likewise, the bacterial transfer was minimal for secondary cross-contamination, which recorded 46.2 MPN/g (from contaminated water to lettuce) and 0.75 MPN/g (from contaminated plate to lettuce).

In conclusions, there is an immediate need for further investigation to look into the widespread of *V. parahaemolyticus* in Malaysia. Continued research, risk assessment and surveillance on the behaviour and characteristics of *V. parahaemolyticus* is very important in order to control, prevent and reduce the emerging problems caused by this interesting bacterium.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PEMANTAUAN *VIBRIO PARAHAEMOLYTICUS* DALAM MAKANAN LAUT BERCENGKERANG YANG DIBELI DARI RUNCIT TERPILIH DI SELANGOR, MALAYSIA

Oleh

MALCOLM TAN TURK HSERN

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Tujuan kajian ini adalah untuk mengoptimumkan assay reaksi polimerasi berantai multiplex (mPCR) untuk mengesan kehadiran *V. parahaemolyticus* dan strain patogenik (*tdh*+ dan/atau *trh*+) masing-masing, dan dibandingkan dengan assay amplifikasi bermediasi-gelung sesuhu (LAMP). Assay-assay ini turut dikombinasi dengan kaedah jumlah paling mungkin (MPN) untuk mengira kepekatan bakteria daripada pelbagai sampel makanan laut bercengkerang yang diperolehi dari pasar borong dan pasar raya besar. Data yang diperolehi sejurusnya diaplifikasikan dalam model penilaian risiko mikrob (MRA) untuk menilai risiko kesihatan umum. Tambahan pula, satu kajian simulasi dapur turut dijalankan untuk medapatkan data kontiminasi bersilang dan dekontaminasi.

Sejumlah 232 sample iaitu kerang, lala dan udang dibeli secara rawak daripada pasar borong dan pasar raya besar di Selangor. Sejumlah daripada 229 (98.7%) sample yang diuji adalah positif dengan *V. parahaemolyticus* berkepekatan dari 30 hingga >110, 000 MPN/g. Tujuh puluh tujuh dari 232 (33.1%) sampel yang diuji didapati positif dengan *tdh*+ *V. parahaemolyticus* berkepekatan dari 30 hingga >110, 000 MPN/g. Manakala, 16 daripada 232 (6.9%) sampel yang diuji positif dengan *trh*+ berkepekatan dari 30 hingga 9,600 MPN/g. Di samping itu, kami mendapati bahawa LAMP adalah lebih robust dan sensitif daripada multiplex PCR.

Kerang dipilih secara selektif untuk MRA disebabkan prevalen strain *V. parahaemolyticus* yang patogenik adalah sangat tinggi dalam sample ini. Kajian ini berasakan experimentasi dan data survey semasa yang dikombinasi dengan @RISK software untuk mengsimulasikan kerawakan dengan mengunakan simulasi Monte Carlo. Estimasi berisiko divaluekan pada 1.04E-4/suapan sehari/seorang. Ini turut boleh ditranslasikan kepada 54 hingga 26,300 kes setahun.

Simulasi mengendalikan kerang dimodalkan berasakan event sebenar di tahap dapur untuk mendapatkan data quantifikasi *V. parahaemolyticus* kontaminasi bersilang kepada makanan yang sedia untuk dimakan (RTE; contohnya, sayur salad). Hasil daripada kajian ini 20.40 ±13.78% daripada jumlah populasi *V. parahaemolyticus* yang dipinda kepada tangan dan peralatan dapur, dan sayur salad mempunyai kepekatan *V.*

parahaemolyticus sebanyak 7432.5 MPN/g. Dekontaminasi berjaya mengurangkan populasi *V. parahaemolyticus* sebanyak 97.63 ± 3.43% (air) and 96.00 ± 5.03% (kain), dan sayur salad hanya mempunyai kepekatan *V. parahaemolyticus* sebanyak 9.27 MPN/g. Di samping itu, bakteria yang dipinda daripada konteminasi bersilang sekunder adalah minimal iaitu sebanyak 46.2 MPN/g (dari air kepada sayur salad) and 0.75 MPN/g (dari pinggan kepada sayur salad).

Konklusinya, siasatan lanjutan diperlukan segara untuk meninjau perubahan semasa *V. parahaemolyticus* di Malaysia. Hasil kajian berterusan, penilaian berisiko, peninjauan semasa dan penilaian karakter *V. parahaemolyticus* adalah sangat penting untuk megawal, mengelak dan mengurangkan masalah yang diakibatkan oleh bakteria oleh bakteria ini.

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I certify that an Examination Committee has met on 16 September to conduct the final examination of Malcolm Tan Turk Hsern on his degree thesis entitled "Surveillance of *Vibrio parahaemolyticus* in Shellfish purchased from Selected Retailers in Selangor, Malaysia" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science

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LIST OF ABBREVIATIONS

µl	-	Microliter
ALOP	-	Appropriate Level of Protection
APW	-	Alkaline Peptone Water
ATCC	-	American Type Culture Collection
BAM	-	Bacteriological Analytical Manual
bp	-	base pair
CDC	-	Centre for Disease Control
Codex	-	Codex Alimentarius Commission
DNA	-	Deoxynucleic acid
dNTP	-	deoxyribonucleoside triphospate
EDTA	-	Ethylenediaminetetraacetic acid
FAO	-	Food and Agricultural Organization
FDA	-	Food and Drug Administration
FSO	-	Food Safety Objectives
GHP	-	Good Hygiene Practices
HACCP	-	Hazard Analysis Critical Control Point
ICMSF	-	International Commission on Microbiological Specifications for Food
LAMP	-	Loop-mediated isothermal amplification
$MgCl_2$	-	Magnesium Chloride
mM	-	milliMolar
MOH	-	Ministry of Health
MPN	-	Most Probable Number
MRA	-	Microbial Risk Assessment
NaCl	-	Sodium Chloride
PCR/mPCR	-	Polymerase Chain Reaction/multiplex Polymerase Chain Reaction
RTE	-	Ready-to-eat
RPM	-	revolution per minute
SOP	-	Standard Operating Procedures
TBE	-	Tris-Boric acid-EDTA
TSA	-	Tryptic Soy Agar
U	-	Unit
U. S	-	United States
UV	-	Ultra violet
WHO	-	World Health Organization
WTO	-	World Trade Organization
α	-	Alpha
β	-	Beta

CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

Seafood is a nutritious food consumed all over the world. As a prime source of high-quality protein, oil-rich and high in mineral, seafood is highly desirable in a healthy diet. Living in the marine environment, these sea creatures are constantly exposed to various pathogens which are detrimental to the publics' health. Thus, prior to the consumption of raw or undercooked seafood, one's life may be at risk. Jacxsens *et al*. (2009) have also reported that consumption of contaminated seafood has led to numerous foodborne outbreaks. Today, *Vibrio parahaemolyticus* is the leading causative pathogen often due to the consumption of raw or undercooked seafood, in which patients were reported to experience severe diarrhea, abdominal cramps, nausea, vomiting, headaches, fever and chills (Shimohata & Takahashi, 2010).

V. parahaemolyticus infection, nevertheless, is common in many Asian countries, inclining to their high seafood diets (Alam *et al*., 2002). For instance, Japan, Taiwan, China, Korea and India are among the major countries that are struggling with this foodborne pathogen (Deepanjali *et al*., 2005, Liu & Su, 2007 & Kim *et al*., 2012). Likewise, cases of *V. parahaemolyticus* are also common in South East Asia, for example, in Thailand and Cambodia (FAO/WHO, 2011 & Vandy *et al*., 2012). Nakaguchi (2011) also noted a high prevalence of *V. parahaemolyticus* in seafood investigated from Malaysia and Indonesia, in which supposed to signify a high infection rate, and thus, suggesting that cases of foodborne illness due to this pathogen are underreported. This is in agreement with the statement by Bilung *et al*. (2005), where infection cases for *V. parahaemolyticus* reported in Malaysia are infrequent. Creditably, the mild infections by *V. parahaemolyticus* are often neglected and pass unnoticed, hence contribute to underestimate the burden of *V. parahaemolyticus* disease, in which a major limitation in foodborne reporting in Malaysia, likewise, for other foodborne pathogens (Soon *et al*., 2011).

Malaysian government acknowledges the rising issue of food safety, and efforts to intervene have been attempted, for instance, in 2002, the National Committee on Risk Analysis (NCRA) was established under the Food Safety and Quality Division, Ministry of Health Malaysia to look into food safety issues in the country. The NCRA initiated the first microbiological risk assessment in 2004 to estimate the risk posed by *V. parahaemolyticus* in black tiger prawn (Mohamad *et al*., 2006). The project was attempted to request for expert advice due to the increased rejection of block frozen raw black tiger prawn by certain importing countries, and also, to issue a safety concern to the public (Sani *et al*., 2013). Besides, attempts to improve the outbreak surveillance and monitoring system, likewise, implementing training (eg, sanitary food handling) and Hazard Analysis Critical Control Point (HACCP) were also part of the government's effort to prevent and/or reduce foodborne diseases. Also, some of the key agencies from the Malaysia's Ministry of Health, academia, industries and research institutions continue to strengthen their collaboration and networking in order to

coordinate the prevention and control of foodborne diseases, in attempts to improve the publics' health (Soon *et al*., 2011).

Basically, this study evaluates the biosafety of *V. parahaemolyticus* in shellfishes, as an effort to provide an insight of Malaysia scenario and would warrant some degree of attention by public health authorities to better understand the potential risk posed by *V. parahaemolyticus*. Considering the fact that microorganisms are "dynamic", and may change drastically over time, constant monitoring is required to ensure our foods are safe for consumption. Therefore, the findings in this study would serve as a useful data and to further refine the food safety industries in Malaysia.

1.2 Objectives

The objectives of this study are:

1. To optimize the multiplex polymerase chain reaction (mPCR) assay for detection of *V. parahaemolyticus* and the respective pathogenic strains (*tdh*+ and/or *trh*+), and to compare with the loop-mediated isothermal amplification (LAMP) assay.
2. To detect and quantify the total and pathogenic strains of *V. parahaemolyticus* (*tdh*+ and/or *trh*+, respectively) in shellfishes from wet markets and hypermarkets, respectively.
3. To estimate the risk of acquiring Vibriosis (*V. parahaemolyticus* infection) from consumption of bloody clams.
4. To investigate the cross-contamination and de-contamination rate of *V. parahaemolyticus* during handling of bloody clams in domestic kitchen based on locals' practices.

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APPENDICES

Appendix A: Media and solution preparation

Media

Alkaline Peptone Water (APW)

Peptone	10.0 g
Sodium Chloride	10.0 g

Suspend 20.0 g of the APW powder in 1 L of purified water and mix thoroughly. Autoclave at 121°C for 15 minutes. Final pH 8.3 – 8.7.

Tryptic Soy Agar 3% sodium chloride (TSA 3% NaCl)

Pancreatic Digest of Casein	15.0 g
Papaic Digest of Soybean	5.0 g
Sodium chloride	5.0 g
Agar	15.0 g

Suspend 40.0 g of the TSA powder and 30.0 g of sodium chloride in 1 L of purified water and mix thoroughly. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder. Autoclave at 121°C for 15 minutes. Final pH 7.3 ± 0.2.

Gel electrophoresis

10X Tris-Borate-EDTA (10X TBE)

Tris-Base	108.0 g
Boric acid	55.0 g
EDTA	9.3 g
Distilled water	1000 ml

For routine electrophoresis, 10X TBE were diluted to 0.5X TBE, with the pH 8.3. Solution can be kept at room temperature.

1.2% Agarose Gel

Agarose	1.2 g
0.5X TBE	100 ml

Heat until the medium boils. Do not overheat.

Ethidium Bromide solution

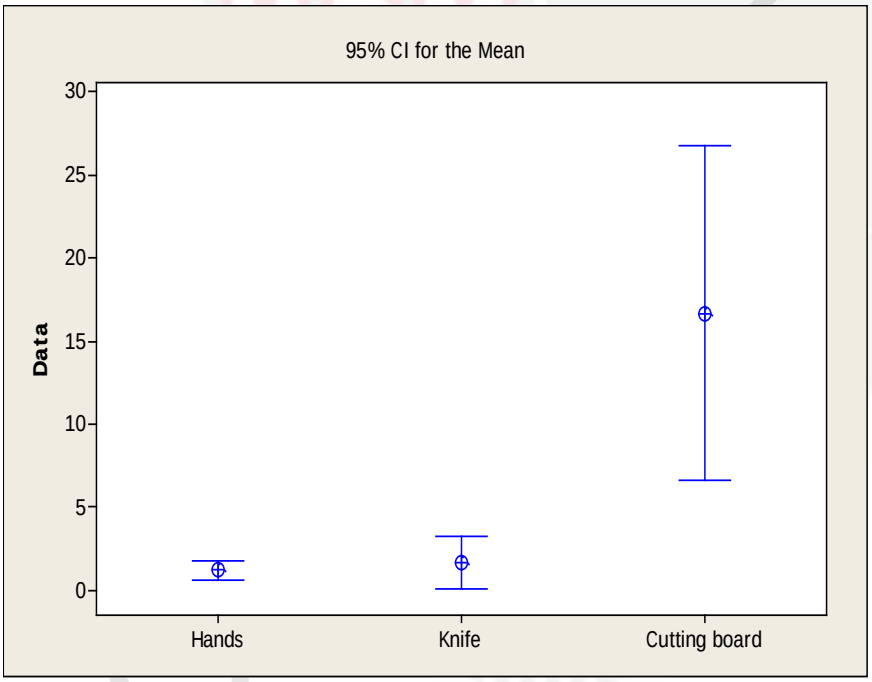
Ethidium bromide	1.0 g
Distilled water	100 ml

Appendix B: Statistical Analysis

Statistical Analysis of Kitchen Simulation Data (Working Chapter 3)
Transfer rate (primary cross-contamination)
Kruskal-Wallis Test: Transfer rate versus surface transferred to

Treatment	N	Median		Ave	Rank	Z
Cutting board	10	14.8000		24.6	4.00	
Hands		10	0.8950		11.4	-1.80
Knife		10	0.8950		10.5	-2.20
Overall		30			15.5	

H = 16.08 DF = 2 P = 0.000
H = 16.09 DF = 2 P = 0.000 (adjusted for ties)



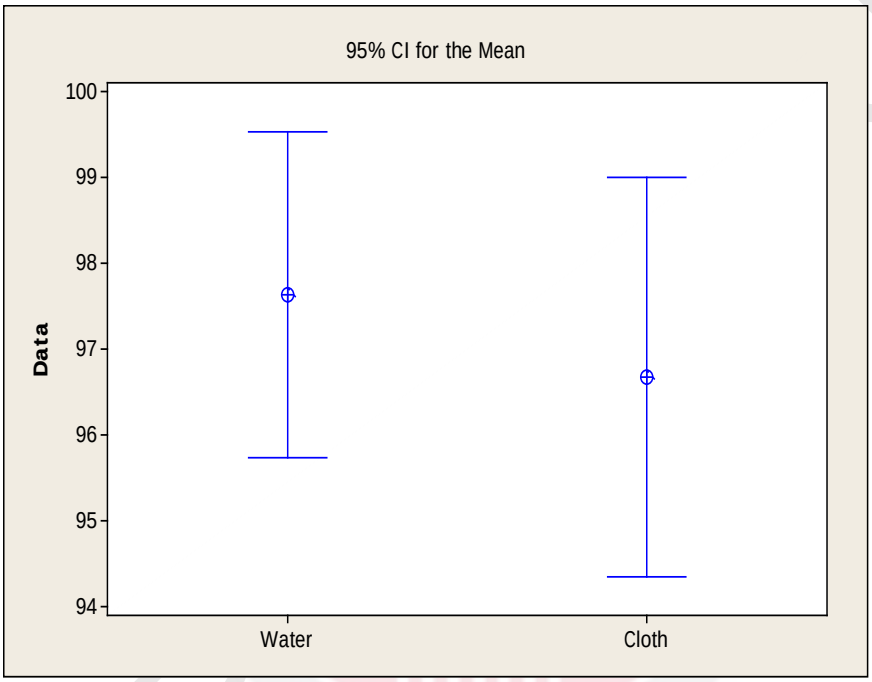
Conclusion: Difference is significant.

Decontamination rate

Mann-Whitney Test: Decontamination rate versus type of treatment

Treatment	N	Median
Water	15	98.790
Cloth	16	98.340

Point estimate for ETA1-ETA2 is 0.430
95.4 Percent CI for ETA1-ETA2 is (-0.679,2.018)
W = 255.0
Test of ETA1 = ETA2 vs ETA1 not = ETA2 is significant at 0.5665



Conclusion: There is no difference between treatments.

Kruskal-Wallis Test: Treatment on hands

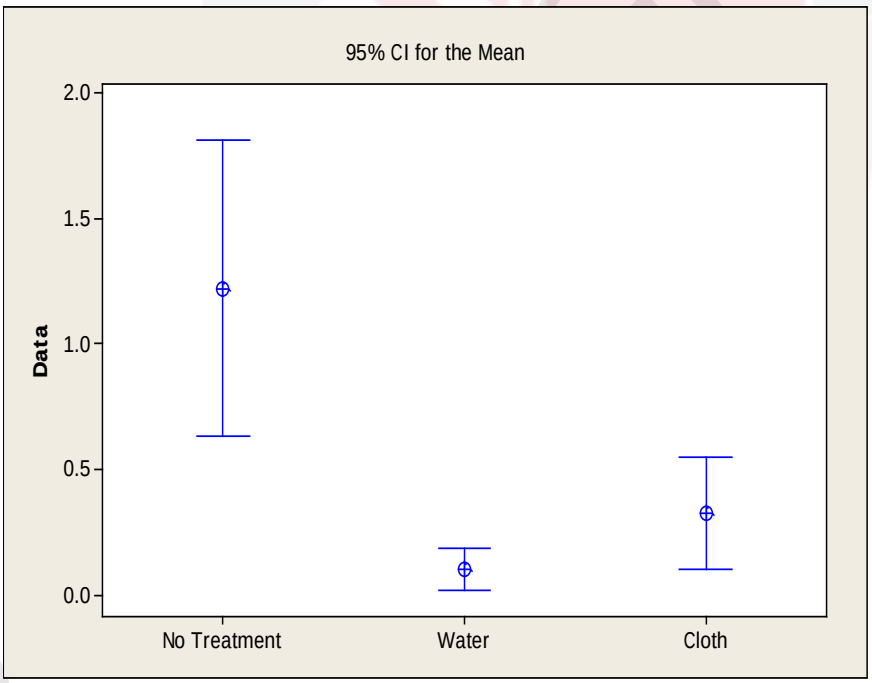
Treatment	N	Median	Ave	Rank	Z
No treatment	10	0.89500		34.6	4.13
Water	15	0.06000		12.9	-3.30
Cloth	16	0.14000		20.1	-0.37
Overall	41			21.0	

H = 19.89 DF = 2 P = 0.000
H = 19.96 DF = 2 P = 0.000 (adjusted for ties)

Mann-Whitney Test: Water versus table cloth

Treatment	N	Median
Water	15	0.0600
Table Cloth	16	0.1400

Point estimate for ETA1-ETA2 is -0.0700
95.4 Percent CI for ETA1-ETA2 is (-0.3400,-0.0002)
W = 192.0
Test of ETA1 = ETA2 vs ETA1 not = ETA2 is significant at 0.0604
The test is significant at 0.0594 (adjusted for ties)



Conclusion: Difference is significant.

Kruska-Wallis: Treatment on knife

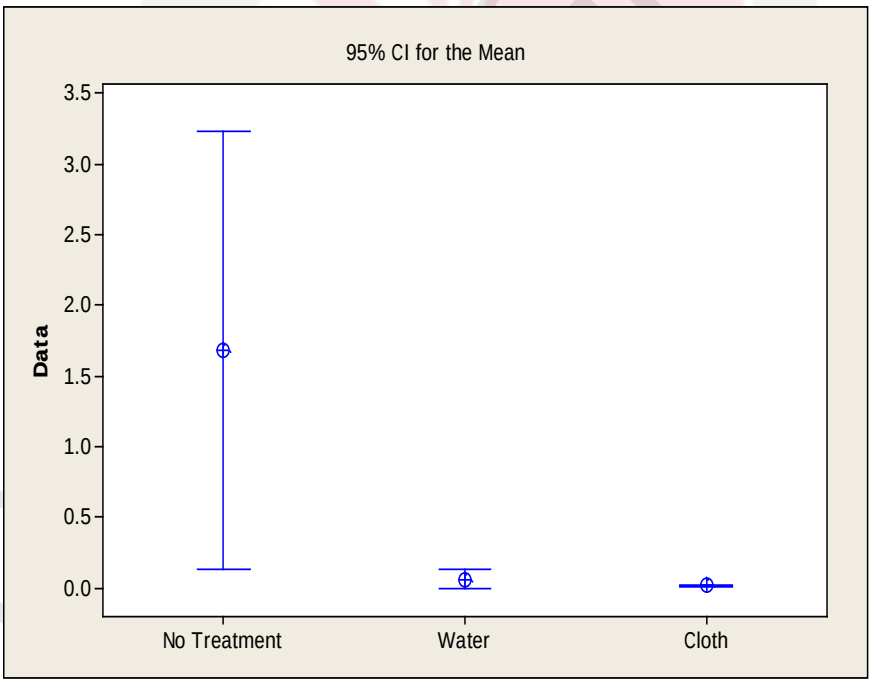
Treatment	N	Median	Ave	Rank	Z
No Treatment	10	0.89500	35.3	4.33	
Water	15	0.01000	18.2	-1.15	
Cloth	16	0.01500	14.8	-2.67	
Overall	41		21.0		

H = 19.35 DF = 2 P = 0.000
H = 19.80 DF = 2 P = 0.000 (adjusted for ties)

Mann-Whitney: Water versus cloth

Treatment	N	Median
Water	15	0.0100
Cloth	16	0.0150

Point estimate for ETA1-ETA2 is 0.0100
95.4 Percent CI for ETA1-ETA2 is (-0.0100,0.0400)
W = 264.0
Test of ETA1 = ETA2 vs ETA1 not = ETA2 is significant at 0.3529
The test is significant at 0.3413 (adjusted for ties)



Conclusion: Difference is significant.

Kruskal-Wallis: Treatments on cutting board

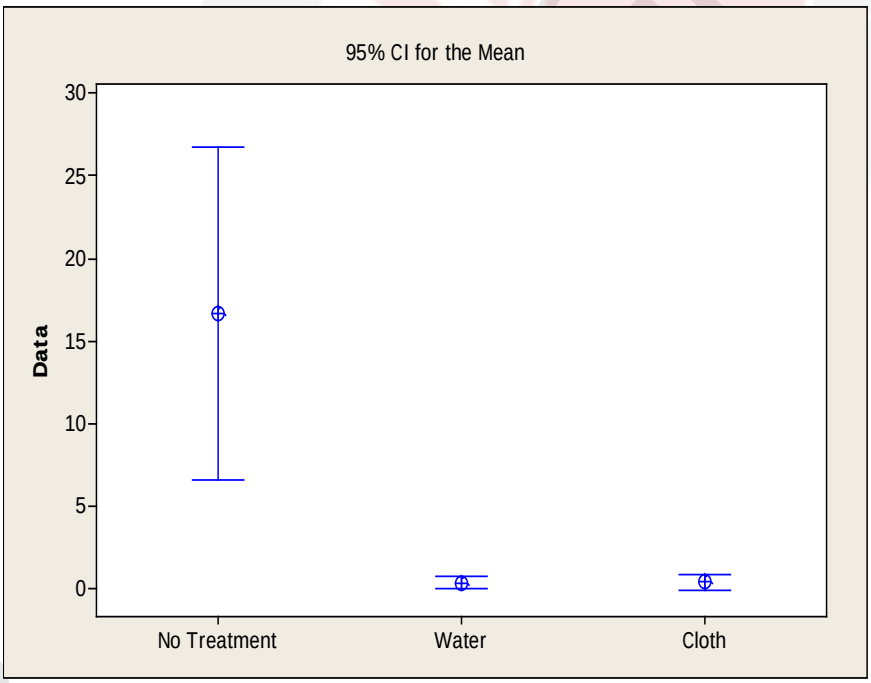
Treatment	N	Median	Ave	Rank	Z
No Treatment	10	0.89500	35.3	4.33	
Water	15	0.01000	18.2	-1.15	
Cloth	16	0.01500	14.8	-2.67	
Overall	41		21.0		

H = 19.35 DF = 2 P = 0.000
H = 19.80 DF = 2 P = 0.000 (adjusted for ties)

Mann-Whitney: Water versus table cloth

Treatment	N	Median
Water	15	0.100
Cloth	16	0.035

Point estimate for ETA1-ETA2 is 0.040
95.4 Percent CI for ETA1-ETA2 is (-0.050,0.180)
W = 278.0
Test of ETA1 = ETA2 vs ETA1 not = ETA2 is significant at 0.1383
The test is significant at 0.1361 (adjusted for ties)

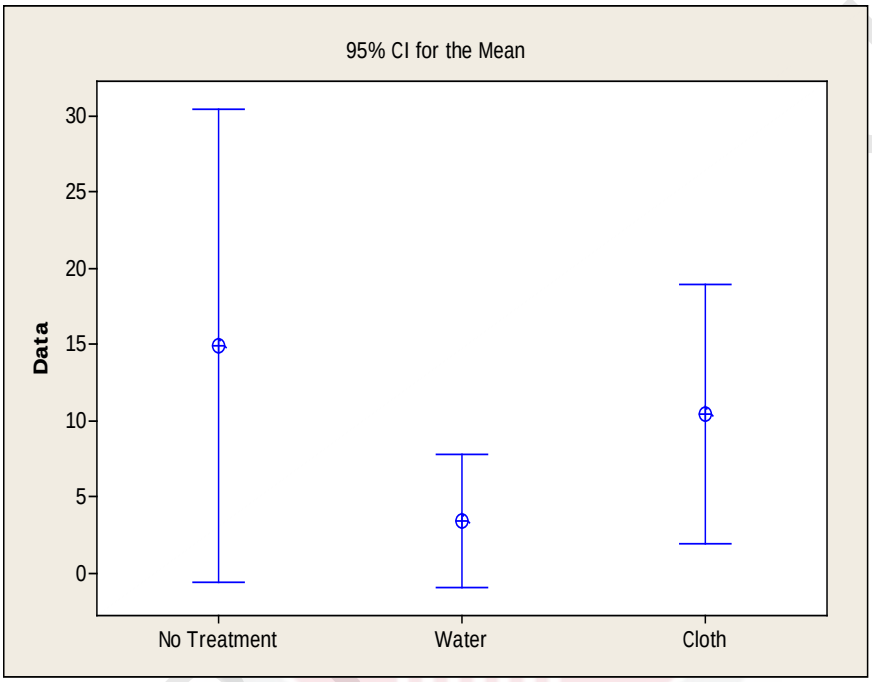


Conclusion: Difference is significant.

Kruskal-Wallis: Transfer rate to lettuce

Treatment	N	Median	Ave	Rank	Z
No Treatment	4	14.385	10.3	1.56	
Water	5	2.860	4.2	-2.20	
Cloth	5	11.580	8.6	0.73	
Overall	14		7.5		

H = 5.19 DF = 2 P = 0.075



Conclusion: There is no difference between transfer rates.

Transfer efficiency (secondary cross-contamination)

Kruskal-Wallis Test: Primary vs secondary cross-contamination

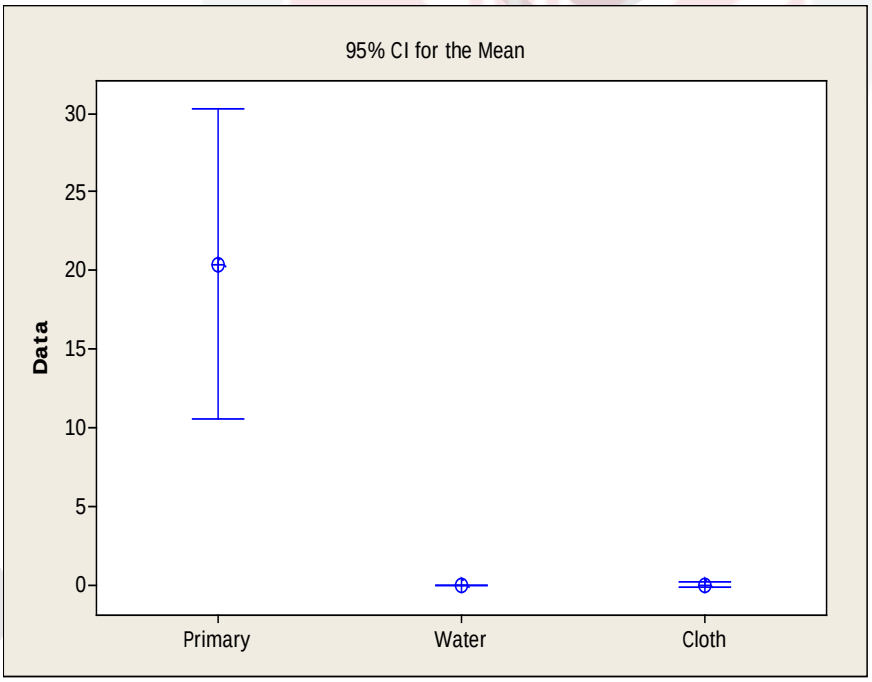
Treatment	N	Median	Ave	Rank	Z
Cloth	11	0.01000	8.0	-3.17	
Primary	10	18.23000	21.5	4.22	
Water	5	0.02000	9.7	-1.24	
Overall	26		13.5		

H = 17.96 DF = 2 P = 0.000
H = 18.25 DF = 2 P = 0.000 (adjusted for ties)

Mann-Whitney Test: Secondary cross-contamination (water versus cloth)

Treatment	N	Median
Water	5	0.0200
Cloth	11	0.0100

Point estimate for ETA1-ETA2 is 0.0100
95.9 Percent CI for ETA1-ETA2 is (-0.0299,0.0200)
W = 48.5
Test of ETA1 = ETA2 vs ETA1 not = ETA2 is significant at 0.5332
The test is significant at 0.5184 (adjusted for ties)



Conclusion: Difference is significant

BIODATA OF THE STUDENT

Malcolm Tan Turk Hsern was born in Selangor, Malaysia on November 24th, 1989. He went for his primary education at Sekolah Rendah Kebangsaan SS17 Subang Jaya, Subang Jaya. After that, he continued her secondary education at Sekolah Menengah Kebangsaan SS17 Subang Jaya, Subang Jaya. He went to foundation in 2007 and pursued his Bachelor of Science (Biotechnology) in 2008 at University Tunku Abdul Rahman (UTAR) and graduated in 2011. He then decided to further study at Universiti Putra Malaysia (UPM) and was offered to pursue a Master's Degree by the School of in 2013. The followings are the publications that he and his colleagues have published and conferences that he has attended during the course of her study.

LIST OF PUBLICATIONS

Malcolm, T. T. H., Cheah, Y. K., Mohamed Radzi, C. W. J. W., Abu Kasim, F., Kantilal, H. K., John, T. Y. H., Martinez-Urtaza, J., Nakaguchi, Y., Nishibuchi, M. & Son, R. (2015). Detection and quantification of pathogenic *Vibrio parahaemolyticus* in shellfish by using multiplex PCR and loop-mediated isothermal amplification assay. *Food Control* 47: 664-671.

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Malcolm T. T. H., Cheah Y. K., Che Wan Jasimah W. M. R., Haresh K. K., Nakaguchi Y., Nischibuchi M., and Son R. Incidence of Pathogenic *Vibrio parahaemolyticus* in Cockles Determined by the Most Probable Number-Multiplex Polymerase Chain Reaction (MPN-mPCR). 2nd International Food Safety Conference 2-3 December, 2013. Royale Chulan Hotel, Kuala Lumpur.