UNIVERSITI PUTRA MALAYSIA

CLINICOPATHOLOGICAL ASSESSMENT OF INTRAMAMMARY ADMINISTRATION OF GENETICALLY ENGINEERED Salmonella agona (4KA32) IN DOMESTIC DOGS

SUJEY KUMAR RAJENDREN

FPV 2018 3

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By

SUJEY KUMAR RAJENDREN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Veterinary Science

November 2017

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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 Veterinary Science

CLINICOPATHOLOGICAL ASESSMENT OF INTRAMAMMARY ADMINISTRATION OF GENETICALLY ENGINEERED *Salmonella agona (4KA32)* IN DOMESTIC DOGS

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November 2017

Chair: Assoc. Prof. Gayathri Thevi Selvarajah, PhD
Faculty: Veterinary Medicine

Salmonella is a good bacterial candidate for anti-cancer therapy because of its characteristics such as motile, facultative anaerobiosis, and the ability to invade epithelial cells and engineered auxotrophies. *Salmonella enterica* serovar Agona is less pathogenic compared to *Salmonella enterica* serovar Typhimurium. *Salmonella enterica* serovar Agona can be genetically engineered to reduce further its pathogenic effects on hosts by eliminating the virulence genes of SopB and SopD. Genetically engineered *Salmonella enterica* serovar Agona (4KA32) was tested on mice model and showed 100% survival rate over two months period. The pathogenicity of 4KA32 in dogs are undefined. The objectives of this research are to determine the preliminary clinical and pathological effects of intra-mammary administration of 4KA32 in seven experimental domestic healthy dogs. Dogs were subjected to either single or repeated dosing over three weeks and euthanized at the end of study period for histopathology evaluation. 4KA32 was administered at various concentrations from 1×10^6 CFU/mL to 1×10^9 CFU/mL for both single and repeated dosing. There was significant increase ($p < 0.05$) of monocyte count across all the dogs at Day 1 and persisted until the end of the study. The monocyte count of dog injected higher doses decreased significantly ($p < 0.05$) when compared to monocyte count of dog injected with dose 1×10^6 CFU/mL. There was significant increase ($p < 0.05$) of neutrophils count across all the dogs at Day 1 which persisted until Day 4. The neutrophil count of dogs injected with single dose of 1×10^8 CFU/mL and 1×10^9 CFU/mL showed significant neutrophilia compared to single dose 1×10^6 CFU/mL. The neutrophils count of dogs injected with repetitive dose of 1×10^7 CFU/mL and 1×10^9 CFU/mL significantly increased compared neutrophils count of dogs injected with dose 1×10^6 CFU/mL. Lymphocytosis was not observed in dogs injected with repetitive dose of 4KA32. Whereas, dogs injected with single dose of 4KA32 showed significant lymphocytosis ($p < 0.05$) on Day 4 only. Comparing lymphocyte count across the concentration, dogs injected with single dose of

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1×10^6 CFU/mL of 4KA32 showed lymphocytosis and lymphocyte count for higher doses significantly decreased ($p < 0.05$). Serial faecal and blood samples were cultured negative for 4KA32 growth. All dogs demonstrated superficial inflammation and swelling on the injected sites which healed naturally by Day 3. The highest concentrations of 4KA32 at 1×10^9 CFU/mL resulted in formation of superficial ulceration on Day 5 that gradually healed within seven days. None of the dogs developed fever or systemic signs including diarrhoea or inappetance during the experimental observation. Histopathology analysis revealed, mild to moderate infiltration of inflammatory cells on mammary gland of dogs injected with dose 1×10^6 CFU/mL to 1×10^8 CFU/mL. Severe infiltration of inflammatory cells was observed with dog injected with dose 1×10^9 CFU/mL of 4KA32. Histopathology of intestine revealed dog injected with 1×10^7 CFU/mL developed mild to moderate ulceration whereas dog injected with 1×10^9 CFU/mL developed moderate to severe ulceration. This study provides evidence that 4KA32 has minimal to none systemic pathogenic effects with potential to induce local inflammation in mammary tissues. Detection of 4KA32 isolates within the lung, mesenteric lymph node and kidney without pathogenic lesions suggest that this bacteria can have potential to target metastasis of cancer cells which is common to these sites. Other tissues were negative for 4KA32. There was minimal pathogenicity observed in all the dogs injected with 4KA32. This suggest that 4KA32 is safe to be administered in dogs.

Keyword: *Salmonella enterica* serovar Agona, dog, mammary gland, pathogenicity

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

PENILAIAN KLINIKOPATOLOGIK DALAM ANJING TEMPATAN SELEPAS PENYUNTIKAN *Salmonella agona (4KA32)* YANG GENETIKNYA DIUBAH SUAI PADA KALENJAR SUSU

Oleh

SUJEY KUMAR RAJENDREN

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Pengerusi: Prof. Madya. Gayathri Thevi Selvarajah, PhD
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Salmonella merupakan bakteria yang baik untuk digunakan sebagai terapi anti-kanser kerana ciri-cirinya seperti motil, anaerobiosis fakultatif, dan berupaya menyerang sel epitelium dan auxotrophies. *Salmonella enterica* serovar Agona boleh diubah secara genetik untuk mengurangkan kesan patogeniknya pada sel hos melalui perubahan pada gen virulensi SopB dan SopD. *Salmonella enterica* serovar Agona (4KA32) yang diubah suai genetiknya, telah dikaji pada modal tikus, dimana ia menunjukkan 100% kadar hidup selama dua bulan. Patogenisiti 4KA32 pada anjing belum dikaji lagi. Objektif penyelidikan ini adalah untuk menentukan kesan klinikal dan patologi awal selepas penyuntikan 4KA32, pada tujuh ekor anjing. Anjing-anjing tertakluk kepada sama ada dos tunggal atau berulang selama tiga minggu dan kemudian dimatikan untuk penilaian histopatologi pada akhir eskperimen. 4KA32 diberikan dengan pelbagai julat dos daripada 1×10^6 CFU/mL hingga 1×10^9 CFU/mL untuk kedua-dua rejim dos. Terdapat peningkatan ketara ($p < 0.05$) bagi jumlah monosit pada kesemua anjing pada hari pertama selepas inokulasi dan ia berterusan hingga ke akhir eksperimen. Jumlah monosit untuk anjing yang disuntik dengan dos tinggi berkurang secara ketara ($p < 0.05$) berbanding jumlah monosit anjing yang disuntik dos 1×10^6 CFU/mL. Jumlah neutrofil meningkat secara ketara ($p < 0.05$) pada semua anjing pada hari pertama dan ia berterusan hingga hari ke-4. Tedapat neutrofilia yang ketara ($p < 0.05$) pada anjing yang disuntik dos tunggal 1×10^8 CFU/mL dan 1×10^9 CFU/mL berbanding dengan jumlah neutrofil pada anjing yang disuntik dos tunggal 1×10^6 CFU/mL. Jumlah neutrofil pada anjing yang disuntik 4KA32 berulang kali dengan dos 1×10^7 CFU/mL dan 1×10^9 CFU/mL menunjukkan kadar peningkatan neutrofil yang ketara ($p < 0.05$) berbanding jumlah neutrofil pada anjing yang disuntik 4KA32 berulang kali dengan dos 1×10^6 CFU/mL. Limfositosis tidak dilihat pada anjing –anjing yang disuntik dos berulang, tetapi anjing yang disuntik dos tunggal 4KA32 menunjukkan limfositosi yang ketara ($p < 0.05$) pada hari ke-4 sahaja. Perbandingan jumlah limfosit untuk semua dos

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menunjukkan limfositosis pada anjing yang disuntik dos tunggal 1×10^6 CFU/mL dan jumlah limfosit pada dos tinggi menunjukkan pengurangan yang ketara ($p < 0.05$) berbanding dos 1×10^6 CFU/mL. Sampel-sampel najis dan darah didapati negatif untuk kultur 4KA32. Semua anjing menunjukkan keradangan dan bengkak pada kelenjar mammari yang disuntik dimana ianya sembuh secara semulajadi pada hari ke-3. Jumlah tertinggi 4KA32 iaitu pada 1×10^9 CFU/mL didapati menyebabkan pembentukan ulser pada hari ke-5 suntikan yang sembuh beransur-ansur dalam hari ke-7. Tiada anjing yang mengalami demam atau tanda-tanda sistemik termasuk cirit-birit atau kurang selera makan semasa pemerhatian eksperimen. Pada pemeriksaan histopatologi tisu, peyusupan sel-sel inflamasi yang sedikit hingga sederhana dilihat pada kalenjar susu anjing yang disuntik dos 1×10^6 CFU/mL hingga 1×10^8 CFU/mL. Peyusupan sel-sel inflamasi yang teruk dilihat pada kelenjar susu anjing yang disuntik dos 1×10^9 CFU/mL 4KA32. Pemeriksaan histopatologi pada usus anjing yang disuntik dos 1×10^7 CFU/mL, menunjukkan ulser yang sedikit hingga sederhana, malah usus anjing yang disuntik dos 1×10^9 CFU/mL menunjukkan ulser yang sederhana hingga teruk. Kajian ini membuktikan bahawa 4KA32 tidak mempunyai sebarang kesan patogenik sistemik yang berpotensi mendorong keradangan dalam tisu-tisu mammari. Pengesanan Salmonella di dalam paru-paru, nodus limfa mesentrik, dan buah pinggang tanpa lesi patogen mencadangkan bahawa bakteria ini boleh berpotensi untuk menyasarkan metastasis sel-sel kanser yang biasa merebak ke organ-organ tersebut. Organ dan tisu lain tidak menunjukan isolasi bakteria tersebut. Kajian ini menunjukkan, patogenik yang minima ditunjukkan oleh 4KA32 dan ia selamat untuk disuntik pada anjing.

Kata Kunci: *Salmonella enterica* serovar Agona, anjing, kelenjar susu, patogenik

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ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my supervisors, Assoc. Prof. Dr. Gayathri Thevi Selvarajah and Assoc Prof. Dr. Cheah Yoke Kqueen for their excellent guidance, caring, patience and providing me with an excellent atmosphere for doing research. I would like to thank Dr. Reuben Sunil Kumar Sharma for letting me use parasite laboratory for running some of my blood samples. Besides that, I would like to convey my gratitude to Dr. Suzanne Khoo, who as a friend, was always willing to help and give his best suggestions. It would have been a lonely lab without her.

I would like to thank my family and friends, who were there, cheering me up and stood by me through the good times and bad. Every challenging work needs self-efforts as well as guidance of elders especially those who were very close to our heart. My humble effort I dedicate to my loving family, whose affection, love, encouragement and prays of day and night make me able to finish my task.

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I certify that a Thesis Examination Committee has met on 1st November 2017 to conduct the final examination of Sujey Kumar Rajendren on his thesis entitled "Clinicopathological Assessment of Intramammary Administration of Genetically Engineered *Salmonella Agona (4KA32)* in Domestic Dogs" 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 Veterinary Science.

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TABLE OF CONTENT

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LIST OF TABLES

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LIST OF FIGURES

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LIST OF APPENDICES

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LIST OF ABBREVIATIONS

TNF-α	Tumour necrosis factor alpha
Lox	Lysyl oxidase
SPI	*Salmonella* pathogenic Island
CMT	Canine mammary gland tumour
OHE	Ovariohysterectomy
PD-1	Programmed death 1
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
T1SS	Type One Secretion System
XLD	xylose lysine deoxycholate
HE	Hektoen enteric
TSI	triple sugar iron
LIA	lysine iron agar
M cells	Microfold cells
PSA	Prostate Specific Antigen
Klk1b22	Kallikrein 1-related peptidase b22
TRAIL	TNF- related apoptosis-inducing ligand
DR4	Death receptor 4
DR5	Death receptor 5
FASL	Fas ligand
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-18	Interleukin 18

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CC chemokine-21	Chemokine (C-C motif) ligand 21
TNFSF14	Tumour necrosis factor superfamily member 14
NK	Natural killer
T3SS	Type three secretion system
CO_2	Carbon dioxide
xg	Times gravity
Na_2HPO_4,	Sodium phosphate
KH_2PO_4,	Potassium phosphate
NaCl	Sodium chloride
NH_4Cl	Ammonium chloride
$MgSO_4$	Magnesium sulphate
$CaCl_2$	Calcium chloride
CFU	Colony forming unit

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CHAPTER 1

INTRODUCTION

Salmonella entericar serovar Agona was first discovered in the year 1952, in Ghana (Hoffmann *et al.,* 2015). It is considered a zoonotic organism, which can cause various infections in humans and animal. Transmission of this serovar from animals to humans occurs by ingestion of contaminated beef, pork, chicken, turkey meat cereals or tea (Michael *et al.,* 2006).

Salmonella sp. which are gram-negative, facultative anaerobes that causes intestinal infections. Surprising, *Salmonella sp.* was observed to infiltrate human tumours (Yoon *et al*., 2017). Presence of lipopolysaccharide in *Salmonella sp.*, induces septic shock (Pawelek *et al.,* 2003). However, some mutants of *Salmonella sp.* did not show signs of virulence when Bacon and colleague injected into mice (Pawelek *et al.,* 2003). *Salmonella sp.* are facultative anaerobic which able to colonise large and small tumours. Tumour microenvironment consist of multi-potent stromal cells, fibroblast, blood vessels, endothelial cell precursors, immune cells and cytokines (Casey *et al*., 2015). Tumours undergo angiogenesis, as tumour cells grow much faster than cells making up the blood vessel, which results in biological changes and adaptive metabolisms such a formation of defective vessels, appearance of apoptotic and necrotic cancer tissues, and emergence of hypoxic areas and heterogeneous tumour cell populations (Wei *et al*., 2008). There are two types of hypoxia identified within tumour microenvironment. One is chronic hypoxia due to limited perfusion of oxygen through tissue and another one is cycling hypoxia due to temporary blockage of tumour blood vessels (Casey *et al.*, 2015). Hypoxia provides vast chances for bacteria to infiltrate the tumour (Wei *et al.,* 2008).

Salmonella sp has property to regress and inhibit tumour growth in a wide range of human and mouse tumours such as B16F10 murine melanoma and human tumour xenograft Lox, DLD-1, A549, WiDr, HTB177, and MDA-MB-231 (Luo et al., 2001; Pawelek *et al.,* 2003). To take advantage of this property, hence the possibilities to genetically modify *Salmonella sp* by inserting prokaryotic expression plasmids using mammalian cells *in vitro* and *in vivo* was explored successfully (Yoon *et al.,* 2017).

Genetically modified *Salmonella enterica* serovar Typhimurium (VNP20009) demonstrated significant reduction in Tumour Necrosis Factor alpha (TNF-α) production which eventually cause septic shock and deletion of *purI* gene lead to tumour specific colonization. This is the only genetically modified *Salmonella sp.* that was tested in canine thus far (Luo *et al.,* 2001). Besides that, there are A1-R strain which is leucine and arginine auxotrophic (Li *et al.,* 2013), ΔppGpp is a avirulent *Salmonella enterica* serovar Typhimurium, which was mutated at

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relA⁻ and *spoT*⁻ (Na *et al.,* 2006) and LH430 which was engineered at *pho*P⁻and *pho*Q⁻ which are endotoxin related genes (Li *et al.,* 2013).

In a study using mice model, *Salmonella sp.* were seen in the necrotic regions and some in the cytoplasm of the tumour cells itself. This observation leads to understanding of *Salmonella sp.* pathogenicity islands to further understand the mechanism of how *Salmonella sp.* infect and regress the tumour (Pawelek *et al.,* 2003).

It is reported that most virulent genes responsible for bacterial pathogenesis are located in Salmonella Pathogenicity Island (SPI) (Karasova *et al.,* 2009). The virulence factors of *Salmonella sp.* such as *sop*B, *sop*D and *pip*D important in salmonellosis causing enteropathogenicity which results in acute inflammatory cell influx, intestinal fluid secretion and enteritis causing clinical diarrhoea (Khoo *et al.,* 2015). Wild type *Salmonella enterica* serovar Agona (WA32) was genetically engineered to silence the metabolite and virulence genes to develop an attenuated strain (4KA32) which shows significant cytotoxic effects on canine mammary tumour cells *in vitro* (Lee *et al.,* 2015). Even though it is tested on canine mammary tumour cell line, the pathogenicity of 4KA32 is poorly understood in dogs. The hypothesis of the study is, 4KA32 should not be able to express any pathogenicity in term of clinical and pathological due to deletion of the virulence gene.

The main justification of this study is to determine the pathogenicity of 4KA32 on healthy dogs. The objectives of this study as follows:

1. To determine the clinical changes on dogs after administration of 4KA32.
2. To study the changes in haematology after the administration of 4KA32.
3. To determine the pathological changes in dogs after administration of 4KA32.

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