UNIVERSITI PUTRA MALAYSIA

ANTI-CANCER ACTIVITIES OF SALMONELLA ENTERICA SEROVAR AGONA IN CANINE MAMMMARY GLAND TUMOUR CELLS IN VITRO

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ANTI-CANCER ACTIVITIES OF *SALMONELLA ENTERICA* SEROVAR AGONA IN CANINE MAMMMARY GLAND TUMOUR CELLS *IN VITRO*

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CERTIFICATION

It is hereby certified that we have read this project paper entitled "**The Anti-Cancer Activities Of *Salmonella enterica* Serovar Agona In Canine Mammary Gland Tumour Cells *in vitro*"** " by Lee Yee Wen and in our opinion it is satisfactory in terms of scope, quality and presentation as partial fulfilment of the requirement for the course VPD 4999 – Final Year Project.

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DEDICATION

To my parents, friends and teachers, who had made me to be who I am

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LIST OF ABBREVIATION/ SYMBOLS

CMT	canine mammary tumour
et al.	and others
HER-2	human epidermal growth factor receptor 2
%	percent
COX-2	cyclooxygenase 2
SPI1	*Salmonella* Pathogenic Island 1
SPI3	*Salmonella* Pathogenic Island 3
siRNA	Small interfering ribonucleic acid
DNA	deoxyribonucleic acid
UPM	University Putra Malaysia
Cm	centimeter
RPMI	Rosewell Park Memorial Institute media
% (v/v)	percentage volume for volume
CO_2	carbon dioxide
^{o}C	degree celcius
Rpm	revolutions per minute
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)
µL	microlitre
DMSO	Dimethyl Sulfoxide
Nm	nanometer
OD	optimum density
CFU	colony forming unit
EDTA	ethylenediaminetetraacetic acid
SD	standard deviation
TSI	Triple sugar iron agar
SIM	Sulphide-indole-motility agar

ELISA	Enzyme Link Immunosorbent Assay
Mm	millimetre
μM	micromolar
>	more than
<	less than
LD_{50}	lethal dose 50
HSP-27	heat shock protein 27

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ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 – Projek Ilmiah Tahun Akhir.

AKTIVITI ANTI-KANSER *SALMONELLA ENTERICA* SEROVAR AGONA DALAM KANSER KELENJAR MAMMARI ANJING *IN VITRO*

Oleh

LEE YEE WEN

2015

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ABSTRAK

Kanser mama kanin merupakan neoplasma yang biasa berlaku pada anjing betina dan mempunyai ciri epidemiologi, klinikopathologi dan biokimia yang serupa dengan karsinoma payudara manusia. Kajian sebelum ini menunjukkan bakteria mempunyai sifat anti-kanser. Salah satu bakteria yang biasa diselidik sebagai agen anti-kanser ialah *Salmonella* Typhimurium. Bagi memastikan keselamatan penggunaan bakteria terapi *in vivo* ini, virulens bakteria perlu diatenuatkan untuk mengurangkan kesan patogeniknya. Baru-baru ini *Salmonella* Agona jenis liar (WA32) telah dijuruterakan gennya supaya metabolit dan virulens gen didiamkan untuk mengembangkan strain terubah suai/teratenuat gen (4KA32) yang boleh diguna dalam terapi kanser. Dalam kajian ini, assai serangan bakteria analisis sel masa nyata (RTCA) telah dijalankan terhadap titisan sel tumor mama kanin (CMT-Stylo) untuk menilai kesitotoksikan bakteria WA32 dan 4KA32 secara bandingan dengan *S. typhimurium* dan doksorubisin. Sel CMT-Stylo ditumbuhkan dalam E-plate selama 24 jam dan kemudian diperlakukan dengan tiga kepekatan, 10^4, 10^5, 10^6 CFU, bakteria atau kawalan (medium atau doksorubisin). Data masa nyata melalui pengukuran impedans, menyatakan indeks sel (CI), suatu parameter kebolehhidupan sel, direkodkan selama 48 jam perlakuan. Pendedahan CMT-Stylo kepada bakteria WA32 dan 4KA32 menyebabkan kesitotoksikan tererti ($p<0.05$) pengurangan indek sel sebanyak 60 -80% berbanding kawalan tidak diperlaku dan sehingga 90% berbanding sel terperlaku doksorubisin. Tiada perbezaan tererti ($p>0.05$) dalam kesitoktosikan sel CMT-Stylo dilihat terhadap perlakuan dengan mana-mana kepekatan WA32 or 4KA32 bacteria. Pemeriksaan mikroskopi menunjukkan pembulatan sel berlaku dalam kumpulan perlakuan pada 48 jam, yang mungkin merupakan suatu akibat apoptosis. Kesimpulannya, laporan ini adalah yang pertama kali menunjukkan kedua-duan *S.* Agona teratenuat dan jenis liar mempunyai kesan sitotoksik nyata terhadap titisan sel tumor mama kanin, CMT-Stylo. Oleh kerana *S.* Agona strain teratenuat menyebabkan kesitotoksikan sama dengan jenis liar, maka bakteria teratenuat ini disyorkan untuk terapi anti-kanser, dengan andaian yang kesan patogen *in vivo*nya adalah minimum. Adalah disyorkan kajian terhadap haiwan makmal dan anjing yang mengidap tumor mama spontan dijalankan pada masa hadapan.

Kata kunci: kanin, tumor mamari, sitotosisiti, *Salmonella* Agona, *in vitro*

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfilment of the course VPD 4999 – Final Year Project.

ANTI-CANCER ACTIVITIES OF *SALMONELLA ENTERICA* SEROVAR AGONA IN CANINE MAMMMARY GLAND TUMOUR CELLS *IN VITRO*

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Canine mammary tumour is the most common neoplasm in female dogs which possesses epidemiological, clinicopathological and biochemical characteristics similar to human breast carcinoma. Previous studies showed that bacteria have anti-cancer properties. One of the commonly investigated bacteria as anti-cancer agent is *Salmonella* Typhimurium. In order to ensure safety of such *in vivo* therapy, the virulence of the bacteria must be attenuated to reduce the pathogenic effects. Recently *Salmonella* Agona, wild type strain (WA32) was genetically engineered to silence the metabolite and virulence genes to develop a genetically modified /

attenuated strain (4KA32) for cancer therapy. In this study, real-time cell analysis (RTCA) bacterial invasion assay was conducted in a canine mammary tumour cell line (CMT-Stylo) to evaluate the cytotoxicity of WA32 and 4KA32 bacteria in comparison with *S.* Typhimurium and doxorubicin. The CMT-Stylo cells were grown in E-plates for 24 hours and then treated with three concentrations of 10^4, 10^5, 10^6 CFU bacteria or control (media or doxorubicin). Real-time data on impedance measurement, expressed as cell index (CI), a parameter of cell viability, was recorded over 48 hours of treatment. Exposure of CMT-Stylo cells to different concentrations of WA32 and 4KA32 bacteria produced significant ($p<0.05$) cytotoxicity and 60% to 80% reduction in e cell index, compared to untreated control, and up to 90% compared to doxorubicin-treated cells. No significant ($p>0.05$) difference in CMT-Stylo cell cytotoxicity was observed towards treatment with any of the WA2 or 4KA32 bacteria concentrations. Microscopic examination revealed cell round-up in the treatment groups at 48 hours, which could possibly be due to apoptosis. In conclusion, this is the first report demonstrating that both attenuated and wild type *S.* Agona strains have significant cytotoxic effects on the canine mammary tumour cell line, CMT-Stylo. Since the attenuated strain produced similar cytotoxicity compared to the wild-type, the attenuated bacteria is recommended for anti-cancer therapy, with the assumption that it has minimal *in vivo* pathogenic effect. It is recommended that studies on laboratory animal and dog with spontaneous mammary tumours be conducted in the future.

Keywords: canine, mammary tumour, *Salmonella* Agona, cytotoxicity, *in vitro*

1.0 INTRODUCTION

Canine mammary gland tumour (CMT) is the most common neoplasm found in female intact dogs, affecting approximately 260 out of 100,000 dogs in the United States annually (Dorn *et al.,* 1968; Moulton, 1990). Mammary tumours primarily developed in middle-aged to older female intact dogs with increased incidence starting from approximately 6 years of age (Perez Alenza *et al.*, 2000). Dogs with malignant tumour were also found to be significantly older than dogs with benign tumours: mean age for malignant tumour is 9 to 11 while 7 to 9 years with benign tumours (Sorenmo *et al*., 2009; Goldschmidt *et al.,* 2001).

Mammary tumours are detected during physical examination and will typically involve the two caudal pairs, where the mammary glands and tissue are observed to be larger (Benjamin *et al.*, 1999; Taylor *et al.*, 1976; Moulton *et al.*, 1986; Bender *et al.*, 1984). Size of tumour, stage of disease and presence of systemic signs varies in dog with CMT. Majority of the dogs with mammary tumour are systematically healthy when diagnosed (Sorenmo *et al.*, 2013).

Staging is usually done prior initiation of therapy which includes complete blood count (CBC), serum biochemistry, three-view thoracic radiography, fine needle aspiration (FNA) of regional lymph nodes and abdominal ultrasonography (Sorenmo *et al.*, 2013).

Tumour size, evidence of lymph node metastasis and matastasis to distant organs are the three factors incorporated for tumour staging in dogs with CMT. This staging is adapted based on the World Health Organization (WHO) tumour staging for human breast cancer (Sorenmo *et al.*, 2013). Generally, dogs with a small tumour size have a significant longer survival but the impact of size of tumour on survival will be insignificant when there is a local lymph node involvement which attribute to a worse prognosis (Kurzman *et al*., 1986).

Surgical excision is the treatment of choice for all mammary tumours except in inflammatory carcinomas where surgery has no value in controlling the disease as it is aggressive (Fossum, 2007). Excision of the tissue allows for histopathology diagnosis and tumour grading. Diagnosis of molecular markers can also be beneficial for selected targeted therapy whereby drugs that target the specific molecule markers can be used upon identification of the markers in the tumour tissue or other biological samples. For example, Herceptin® is a targeted drug that can down-regulate HER-2 cell surface expression of the human breast cancer and CMT cells (Ku *et al.*, 2008). Adjuvant chemotherapy such as combination of cytotoxic drugs like doxorubicin, carboplatin and lomustine are often administered to reduce risks for metastasis or local recurrence. However, most evidence of the efficacy of adjuvant chemotherapy is weak while hormonal therapy was shown to be ineffective (Sorenmo *et al.*, 2013).

The use of bacteria as a therapeutic agent against tumour is an area with great potential in cancer therapy for both human and animals. However, it is also a challenge as bacteria can cause undesirable pathological effect to the normal tissues in the body (Yu *et al.*, 2012). Hypoxic regions of a solid tumour makes them resistant to many treatments and therefore it has been proposed that strategies to administer anaerobic bacteria to tumours may enhance the cytotoxic effects at these regions and cause tumour regression (Pawelek *et al.*, 1997).

Domestic dogs became the model of choice for novel anti-tumour therapies as they are relatively outbred, immunocompetant with spontaneous tumour development and the histotypes biology and tumour size closely resemble to those in humans. Furthermore, the rapid time course of disease progression as compare to human allow more rapid assessment of therapeutic end points to be done (Thamm *et al.*, 2005) .

Salmonella enterica serovar Agona which is genetically silenced to knock out the virulence genes critical in the pathogenesis of disease in humans and animals has been successfully enhanced as tumour reduction agent in various *in vitro* and *in vivo* studies on cancer models such as liver, colon and head and neck cancer. However, the development of *Salmonella* Agona as a tumour targeting agent would require extensive research prior to testing in *in vivo* models and proven with clinical success may require years of controlled research as compared to the use of *Salmonella* Typhimurium which has already been proven to have high affinity to tumour cells *in vitro* and *in vivo* in numerous investigations. Furthermore, *Salmonella* Agona has lower pathogenicity and able to target tumours making it to be a good candidate to be used for anti-tumour therapy in dogs and cats. The mammary gland tumour has a high prevalence among Malaysian pet dogs (Selvarajah, 2015, personal communication) and hence these dogs can be potentially used for this study.

The objective of this research is to determine the anti-cancer activity of *Salmonella* Agona in canine mammary tumour cells – CMT-Stylo and early passages of primary mammary gland tumour cells in culture.

This research is done based on the hypothesis that *Salmonella* Agona treatment *in vitro* on canine mammary gland cancer cells result in cytotoxic effects by reduction of cell proliferation and increased cell death.

2.0 LITERATURE REVIEW

2.1 Canine mammary gland tumour

2.1.1 Epidemiology and Risk Factor

Mammary neoplasia is the most common malignant neoplasm in bitch with an annual incidence rate of 198 dogs per 100,000 dogs (Schneider, 1970). Another study from the survey of Alameda and Contra Costa counties reported an estimated of approximately 257 malignant mammary tumour per 100,000 in intact bitch (Dorn *et al.*, 1968). In an insurance-based study done by Dobson *et al.* in the year 2002 the annual incidence rate of CMT in the United Kingdom was 205 of 100,000 dogs. In addition, there was a prevalence of 39% CMT diagnosed at the histopathology laboratory of the Faculty of Veterinary Medicine, University Putra Malaysia, from the year of 2006 to 2012 (Kabiru *et al.*, 2012). The incidence rate of CMT varies in different geographical regions depending on the characteristics of the source population and the practice of performing ovariohysterectomy at younger age (Sorenmo *et al.*, 2013). Despite the difference in various geographical regions, the incidence of CMT remains high in female dogs.

More than 70% of the intact females can have more than one tumour at the time of diagnosis (Sorenmo *et al.*, 2013). There are 3 main risk factors for mammary tumour development namely increased hormonal exposure, age and breed. Other risk factors diet, body weight and obesity also contribute to the increased risk but at a lesser degree compared to the aforementioned factors (Sorenmo *et al.*, 2013).

Mammary tumour normally affects middle age to old dogs and the risk increases with age (Schneider, 1970). Besides that, Sorenmo *et al.* also reported that the dogs with malignant tumour have a mean age of 9 to 11 while dogs with benign tumour have a mean age of 7 to 9 years old. Since larger breed dogs have a shorter life span than smaller breed dogs, therefore they also tend to be younger when they are diagnosed with the CMT. Hormonal exposure also plays an important role as the risk of developing mammary tumour is only 0.5% if a dog is spayed before the first estrus and this risk increases with each additional cycle (Schneider, 1969). Similarly, exogenous exposure to hormones oestrogen and progestin will also increase the risk of developing mammary tumour in dogs (Sorenmo *et al.*, 2013). Next, mammary gland tumour also tends to be common in small breed dogs with purebred dogs to be commonly affected. In one study done in the University of Veterinary and Pharmaceutical Sciences, Brno, Czech Republic, the poodles, English cocker spaniels and the Dachshunds had a statistically significant relative risk of developing benign and malignant tumours of the mammary gland (Zatloukal *et al.*, 2005).

2.1.2 Diagnostic methods and staging

Mammary gland tumours are generally detected via thorough routine physical examination (presence of lumps and nodules at the mammary gland and enlargement of the lymph nodes) and the typical signalment and history of the dog such as the age, sex, neuter status, age of neuter, breed, reproduction history, and exogenous hormone administration. Size of tumour, stage of disease and presence of systemic illness vary widely while signs like dyspnoea, cough and gait disorders may develop as a result of metastasis (Sorenmo *et al.*, 2013; Cassali *et al.*, 2007). A baseline data such as complete blood count, serum biochemistry, three-view thoracic radiography are usually obtained due to the risk of metastasis in CMT. Fine needle aspiration of regional lymph node and abdominal ultrasound may be indicated when the lymph nodes involvement are suspected. In addition, mammary tumour cytology can be performed to help rule out non mammary dermal and subcutaneous tumour such as lipoma and mast cell tumours. Fine needle aspiration cytological examination is a simple, rapid and cost effective method with good diagnostic accuracy when applied to mammary gland lesion (Shafiee *et al.*, 2013). Tumours with active ulcerations and discharge from the affected glands' teats can also be evaluated on cytology by obtaining the impression smear of the ulcerated part or from the discharges. The correlation between cytopathology and *ex vivo* histopathology were reported to be between 67.5% to 93% (Cassali *et al.*, 2007; Simon *et al.*, 2009). In a similar study, the sensitivity and specificity for the diagnosis of malignancy using cytological findings were relatively high (86% and 96%, respectively) (Simon *et al.*, 2009).

Diagnostic imaging such as the three view radiography of the lungs (ventral dorsal, right lateral and left lateral), computed tomography (CT) scan and abdominal ultrasonography are useful diagnostic tools to detect distant metastasis at the lymph nodes, liver, spleen, lungs and bone (Sorenmo *et al.*, 2013).

The classification of the canine and feline mammary tumour is commonly done with reference to the "International Histological Classification of Tumours of Domestic Animals" published by the World Health Organization in the year of 1974 and 1999 and a revised system for dog that was published in 2011 (Goldschmidt *et al.*, 2011).

2.1.3 Therapy of canine mammary tumour

Surgical means of removing the mammary tumour is unlikely to result in a cure if multiple glands are involved and may lead to metastatic spread, however surgical interventions on the primary tumours appears to play a role in reducing the recurrence and survival time (Moore, 2006). The extent of surgery for CMT can range from a simple lumpectomy, mastectomy, regional mastectomy, chain mastectomy or staged bilateral mastectomy (Sorenmo *et al.*, 2013). Furthermore, surgical excision allows histological diagnosis and staging to be done which may modify the disease progression. However, the surgical excision of an inflammatory adenocarcinoma is not recommended due to the aggressiveness of this tumour and surgery will not be curative or palliative to the condition (Fossum, 2007).

Chemotherapy is often administered in dogs with CMT to reduce the risk of metastasis or recurrence as an adjuvant to surgery. Dogs with a stage III and IV tumour show significant survival benefit when receive 5-fluororacil and cyclophosphamide adjuvant to surgery compared to dogs treated with surgery alone (Karayannopoulou *et al.*, 2001). Chemotherapy drugs will also cause various side effects and toxicity in dogs and cats and one must take these unique toxicity features into consideration when deciding to administer chemotherapy drugs. For example, cisplatin causes acute vomiting, nephrotoxicity and ototoxicity to dogs while in cats, it will result in fatal pulmonary oedema; doxorubicin cumulative doses can potentially cause irreversible cardiotoxicity in dogs and nephrotoxicity in cats;

lomustine, is hepatotoxic and will cause cumulative thrombocytopenia and delayed myelosuppression; vincristine sulphate will potentially cause peripheral neuropathy (Rodriguez *et al.*, 2010).

The level of pain in a mammary gland tumour can range from none to severe; an inflammatory carcinoma is usually very painful causing pain in 100% of the cases. Therefore, non-steroidal inflammatory drugs (NSAIDS) are usually indicated for the treatment of cancer pain (de Lorimier and Fan, 2005). Besides that, NSAIDs also has anticancer and chemo-preventive effect as overexpression of COX-2 is known to occur in many human and canine tumour including many carcinoma. Selective NSAIDs such as meloxicam works by targeting the COX-2 in cancer cells (de Lorimier and Fan, 2005).

Radiation therapy is seldom utilized for mammary gland tumours but it could be used to help control local disease for incompletely resected tumours or use for non-resectable and inflammatory carcinoma (Novosad, 2003).

In human breast cancer, hormonal therapy is accepted as a treatment option in estrogen receptor positive breast tumours. tamoxifen, an antiestrogen was investigated in a study in human breast cancer resulting in desired tumour response however another report suggests conflicting result (Allen and Mahaffey, 1989; Tavares *et al.*, 2010) Besides that, there are reported side effects to tamoxifen such as vulvular swelling, vaginal discharge and pyometra observed in dogs (Kitchell & Fidel, 1992; Morris *et al.*, 1993).

2.2 *Salmonella* as anti-cancer treatment

Salmonella within the *Enterobacteriacae* family is a group of Gram-negative, facultative anaerobic and facultative intracellular pathogenic bacteria. The genus of *Salmonella* is categorized into two species namely *Salmonella bongori* and *Salmonella enterica* and is further divided into six subspecies (Tindall *et al.*, 2005). Over 2500 of serovars are classified by serotyping and majority of these serovars (60%) belong to *Salmonella enterica* subspecies *enterica* (Poppoff and Le Minor, 1997).

Salmonella colonizes solely the intestinal epithelium leading to gastroenteritis and spread to the liver and spleen causing typhoid fever depending on the serotypes and host. In humans, *Salmonella* Typhimurium causes gastrointestinal infection in the immnunocompromized individuals as well as the elderly and the infant, while in mice it causes enteric fever. *Salmonella* Agona also causes diarrhoea, fever and abdominal cramps especially in infants, elderly and the immunocompromised (CDC, 1998). In the past few years, there is an increasing number of *Salmonella* Agona isolates from infections in human and animals such as cattle, swine, chicken and turkey. Besides that, milk, snacks and vegetarian food were also identified as the souce of infection (Chen *et. al.*, 2009).

The majority of the virulence factors responsible for bacterial pathogenesis such as invasion, intramacrophage survival and extraintestinal spread are encoded by genes termed as the *Salmonella* Pathogenicity Islands (SPI) (Chorobik *et al.*, 2013; Chai-Hoon *et al.*, 2015). *Salmonella sp.* has the ability to multiply in phagocytic and non-phagocytic cells such as the macrophages, dendritic cells, neutrophils, M cells and epithelial cells (Malik Kale *et al.*, 2011). *Salmonella* induces apoptosis and this depended upon the SPI1 effector protein, SipB, to be delivered to the host cell by SPI1 encoding Type III Secretion System (T3SS) (Jesenberger *et al.*, 2000)

The ability of *Salmonella* to grow in aerobic and anaerobic conditions provides an advantage as potential anticancer vector. Wild type of *Salmonella* has been shown to be able to invade tumour tissue *in vitro* and *in vivo*, but may result in the death of the host. Therefore, attenuation of the virulence genes and selected pathogenic components of the bacteria is essential to minimize the potential septicaemia and harm to host as well as demonstrate its usefulness as a potent anti-cancer agent (Pawalek, 1997).

Previous reports have shown that *Clostridium*, *Bifidus* and *Salmonella* are able to accumulate specifically in tumours and *Salmonella* are preferred in bacterial therapy because they are facultative and not obligate anaerobes that can be administered in their motile, vegetative form and therefore target tumour metastases smaller than 200 µm in diameter (Kasinskas and Forbes, 2006). It is also shown that *Salmonella* evokes immune sensitization and anti-tumour immune response by inducing neutrophil infiltration into tumour (Lee *et al.*, 2008).

Attenuation of the virulence on *Salmonella* is a crucial step for the development of *Salmonella*-based vector strains to elicit appropriate immune response and up to about 50 genes of *Salmonella spp.* has been proven to be feasible for inactivation by laboratory selection, site directed or chemical mutageneis (Chorobik *et al., 2*013).

Several attenuated *Salmonella* strains have been developed and published for tumour targeting studies. The VNP20009, which has *pur*I and *msbB* gene deletion, has been used for gene targeted pro-drug therapy and tested for oral delivery and clinical trial. *Salmonella* Typhimurium ΔppGpp strain that is defective in guanosine 5'-disphosphate-3'-diphosphate (ppGpp) has been shown to be effective against murine colon CT-26 tumour. The YB1 strain derived from *Salmonella* Typhimurium strain SL7207 were also able to grow within the tumour and retards its growth. The STM3120 strain with *attenuated* SPI-3 gene showed a reduction in tumour fitness, effective in human prostate PC-3 tumour and can be administered orally (Yu *et al.*, 2012). The A1-R, a leucine and arginine auxotrophic strain derived from ATCC14028 *Salmonella* Typhimurium were shown to have an increased ability to attach to human breast tumour, orthotopic prostate tumour, orthotopic human pancreatic cancer, orthotopic spinal cord glioma, lung fibrosarcoma,and lung osteosarcorma cells and result in significant tumour growth inhibition and complete regression when tested with numerous tumour xenographs in nude mice (Chorobik *et al.*, *2*013).

Effective delivery of drugs into tumour tissue is also one challenging issue in anticancer therapy. An effective prodrug will have to encapsulate the drugs, deliver to the target tissue and release of drug efficiently. Engineering bacteria to deliver chemotherapeutic drugs has been proposed but has yet to develop into practical application. The prospect of using bacteria as nanoparticle carrier that specifically target tumour tissue is highly promising (Forbes, 2010). Bacterially derived *Salmonella* minicells labelled with tumour-sepcific antibody receptors have successfully delivered doxorubicin (MacDiarmid *et al.*, 2007) and siRNA (MacDiarmid *et al.*, 2009) to implanted breast tumour in mouse models. The therapeutic effectiveness of *Salmonella* was shown by successfully targeting a MTDH/AEG-1 based DNA vaccine in orthotopic implanted 4T1 breast cancer tumours that resulted in enhanced doxorubicin activity which inhibited tumour growth and metastatic spread (Qian *et al.*, 2011). These advances have provided new hopes to successfully treat advance and metastatic tumours.

3.0 MATERIALS AND METHODS

3.1 Animal and Cell line

Canine mammary tumour cell line, CMT-Stylo was established from a tumour biopsy derived from a 13 years old, spayed local breed dog with a mammary mass approximately 15cm in size and with evidence of metastasis to liver and spleen. The dog did not receive any chemotherapy prior to removal of mass surgically and survived 3 months post-mastectomy without any chemotherapy. Histopathology diagnosis was mammary gland adenocarcinoma. Cells were brought into culture and passaged for over 30 passages to produce a stable cell line.

Canine mammary tumour cell line CMT-Stylo was obtained courtesy from Prof. Dr. Rasedee Abdullah (Faculty of Veterinary Medicine, University Putra Malaysia, Serdang) and Dr. How Chee Wun (Institut Biosains, UPM, Serdang). The cell lines are maintained and grown as monolayers in a 75cm^2 flask with complete growth media containining RPMI media (Sigma-Aldrich, USA) supplemented with 10% (v/v) heat–inactivated Fetal Bovine Serum (Sigma-Aldrich, South America) and 1% (v/v) Penicillin/Streptomycin (Gibco, USA). Cells were incubated and maintained at 37 ℃, 5% Carbon dioxide in a humidified chamber.

3.2 Primary Mammary Gland Tumour Cell establishment

Tumour biopsies from 2 dogs: age 10 and 14 years old were obtained upon surgical resection and with consent from the clients. Both dogs were presented to the Universiti Veterinary Hospital, UPM for routine diagnosis and therapy. The first dog was presented with a pedunculated mass approximately 5 cm in diameter on the right caudal thoracic gland with no other signs of metastasis. The second dog was presented with a firm mass approximately 10cm in diameter and on the right caudal abdominal gland, with small firm nodules palpable on the left caudal thoracic gland, left cranial abdominal gland, left caudal abdominal gland and bilateral inguinal lymph node were enlarged. Both dogs did not undergo pre- or post surgical chemotherapy interventions.

The tumour biopsies obtained from the dogs were immediately placed in sterile Falcon tubes containing sterile phosphate buffer saline (PBS) (Gibco Life technologies, USA). The tumour tissues were then transferred to a clean Petri dish containing 70% ethanol and then washed twice with sterile PBS before further cutting into pieces less than 3 mm cubes with a sterile scalpel. The dissociation of the cancer cells were conducted by putting the cut tissues into a 15ml tube containing Collagenase Type I (Sigma-Aldrich) and RPMI-1640 medium (Sigma-Aldrich, USA) and vigorously shaked until the cells disrupted and dissociate. The cell suspension was then centrifuged at 5000 rpm for 5 minutes and the supernatant was obtained and spin for another 5 minutes at 5000 rpm. The supernatant was discarded and the cells were then re-suspended with fresh growth medium containing 90% RPMI, 10% Fetal bovine serum and 1% Penicillin/ Streptomycin and incubated further at 37^{o}C under 5% carbon dioxide in a T75 culture flask.

3.3 MTT assay

The cell proliferation of the CMT-stylo cell lines was characterized using the MTT assay (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide), a yellow tetrazole assay. A 200 µL of cell suspension containing 6000, 5000, 4000, 3000, 2000, 1000 cells were seeded into 96-well microplate (Cellstar,USA) and allowed to attach to the bottom of the well. Plates were incubated in a 5% Carbon dioxide incubator at 37^{o}C for 0 hours, 24 hours, 48 hours and 72 hours. A total of 20 µL of MTT reagent (Sigma-Aldrich,USA) from a 5mg/ml stock solution were introduced in each well. The intact mitochondrial reductase converted and precipitated MTT as purple crystals during a 3 hour incubation period. After 3 hours the precipitated crystals were dissolved in 150 µL of DMSO solubilisation solution (Fisher Scientific, USA). The reduced MTT was spectrophotometrically analyzed at 570 nm, using the 630 nm reference of an ELISA reader (Biotek Instrument Inc., Winooski VT, USA).

3.4 Bacterial culture

Wild type *Salmonella enterica* serovar Agona (WA32) and a genetically modified strain with metabolite and virulence genes knock-out (4KA32) was obtained courtesy from Assoc. Prof. Dr. Cheah Yoke Kqueen and his research group (Faculty of Health and Medical Sciences, Universiti Putra Malaysia). The bacteria were grown in xylose lysine deoxycholate (XLD) selective agar at 37^{o}C. Bacteria colonies were diluted and counted using standard protocols for bacteriology laboratory practices. For bacterial invasion assay, bacterial in late log-phase growth (OD_{600nm} = 0.9-1.0) were washed three times and diluted with PBS at concentration of 10^4 CFU, 10^5 CFU and 10^6 CFU.

3.5 Real- time cellular analysis (RTCA) bacterial invasion assay

The iCELLigence™ is a microelectronic biosensor system for cell-based essay that provide dynamic, real-time, label-free cellular analysis for a variety of research such as drug development, toxicology, cancer, medical microbiology and virology. This Real-Time Cellular Analysis (RTCA) technology allows data throughout the entire time course of an experiment and physiological relavant data to be captured. The system consists of an electronic analyzer that fits in the cell culture incubator with an iPad which runs the software and operates the system in wireless mode. The electronic plates (E-Plates L8) which will be attached to the system have microelectrode at the bottom of the wells. The presence of cells on top of the electrodes will affect the local ionic environment leading to increase in electronic impedance. The electrode impedance, which is displayed as Cell Index (CI) can be used to monitor cell viability, number, morphology and extent of adhesion in a number of cell-based assay.

Canine mammary tumour CMT-Stylo cells were harvested using 0.5% Trypsin-EDTA (Gibco, USA) solution followed by inactivation with RPMI 1640 (ScienCell, USA). The cells were then centrifuged at 1200 rpm for 5 minutes, the supernatant was discarded and the cells were resuspended with new RPMI with 10%

Fetal Bovine Serum (Sigma-Aldrich, non USA origin). The cells were then counted with a haemocytometer and seeded into two 8-well RTCA E-Plates L8 (ACEA Bioscience, USA) at a density of 25,000 cells per well. Using the RTCA iCELLigence^{TM} analyzer (ACEA Bioscience, USA), cell index (CI) measurement was collected every 15 minutes. Cells were allowed to grow in the E-plates within the iCELLigence^{TM} analyzer without treatment for 24 hours post seeding at 37^{o}C in a humidified atmosphere containing 5% carbon dioxide. When the cells are in logarithmic growth phase, the cells were treated with *Salmonella* Agona at various concentrations: 10^4 CFU, 10^5 CFU, 10^6 CFU and monitored for every 15 minutes up to 72 hours with iCELLigence^{TM} software. Cells treated with doxorubicin hydrochloride (Boryung Pharmaceutical Co., Ltd., Korea) were used as control and monitored in parallel with the *Salmonella* Agona treated cells. Cell sensor impedance was expressed as an arbitrary unit called the Cell Index (CI). The CI at each time point was defined as $(R_n-R_b)/15$, where R_n is the cell electrode impedance of the well and the R_b is the background impedance of the well with the media alone. Growth curves were normalized to CI at the last measurement time point before compound addition for each well. The cell index of each treatment was analyzed using the RTCA data analysis software 1.0 (ACEA Biosciences, USA). Morphology of the cells in the plate before and after treatment was also captured with an inverted microscope (Carl Zeiss, Primo Vert, Germany)

3.6 Bacteria identification

Dark coloured bacterial colonies from the XLD selective agar for both Wild-type *Salmonella* Agona (WA32) and genetically modified *Salmonella* Agona (4KA32) were grown in blood agar at 37^{o}C for 24 hours. Biochemical tests for gram negative bacteria such as oxidase, triple sugar iron agar (TSI), urease production, sulphide-indole- motility (SIM) and citrate tests were performed to confirm the bacteria up to the species level.

3.7 Statistical analysis

One-way ANOVA tests were used to determine the statistical significance between the control group and the treatment groups. A $p<0.05$ was considered significant for all analyses. Data were expressed as mean ± SD where applicable. All statistical analyses were carried out using the Statistical Package for Social Science (SPSS), version 20.0 (SPSS, IBM Inc., USA). Graphs were drawn on Microsoft Excel version 2007.

4.0 RESULTS

4.1 Establishment of primary cell culture

The primary cell cultures for both times were contaminated and as a result, the tumour cell flasks were discarded.

4.2 Bacteria identification

The wild type *Salmonella* Agona (WA32) colonies isolates were approximately 2-3 mm in size, moist and raised on blood agar. They are non-haemolytic on blood agar. The bacteria is oxidase (-), citrate (+), TSI showed alkaline slant, acidic butt and blackening of the medium as a result of hydrogen sulphide production. The SIM test showed positive sulphur production and negative for indole test. Agglutination was formed when mixed with poly-H antiserum.

The genetically modified *Salmonella* Agona (4KA32) colonies isolates were approximately 2-3mm in size, moist and raised on blood agar. They are non-haemolytic on blood agar. The bacteria is oxidase (-), citrate (-), TSI showed alkaline slant, acidic butt and blackening of the medium as a result of hydrogen sulphide production. SIM test showed positive sulphur production and negative for indole test. Agglutination was formed when mixed with poly-H antiserum.

From the biochemical testing, we can confirm the bacteria isolates are *Salmonella enterica*. No serotyping of bacteria was performed in this experiment.

4.3 MTT proliferation assay

The growth curves of the CMT-Stylo cell lines were analyzed employing the MTT proliferation assay for a better characterization of the proliferative activity of the cells. The wells in the 1000 cells were used for the calculation of the cell doubling time. The cell doubling time intends to provide a rough guideline for the initiation of treatment of *Salmonella* Agona for the RTCA cytotoxicity analysis. The result of the doubling time was shown in Figure 1. The doubling time for the CMT-Stylo cells was 20.38 hours. This doubling time enable us to obtain information on the cell growth characteristics. This information also provides information on the time to initiate the treatment *Salmonella* and chemotherapeutic drugs for this study.

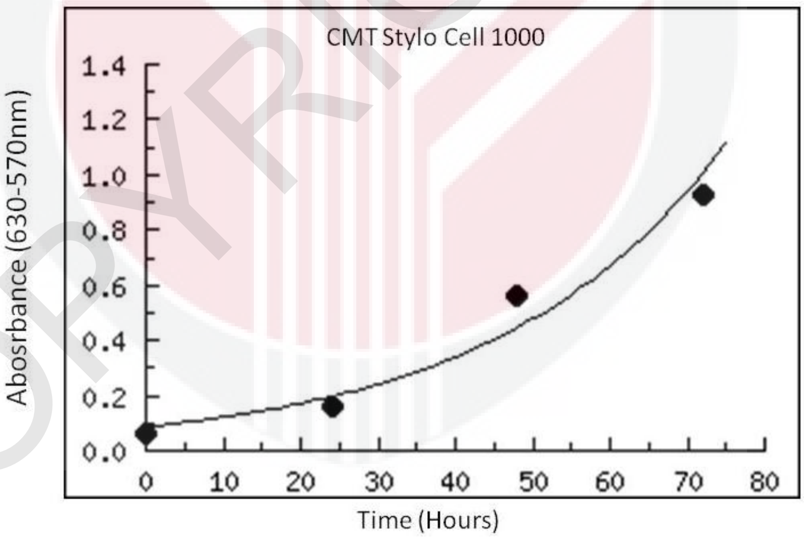

Figure 1. The doubling time of the CMT-Stylo cells in wells seeded with 1000 cells per well.

4.4 Time dependent cytotoxicity

To investigate the temporal dynamics of the cell viability, the iCelligence system was employed to obtain real-time data on the CMT-Stylo cellular growth/ cytotoxicity kinetics up to 48 hours with different concentration of wild type *Salmonella* Agona (WA32) and genetically modified *Salmonella* Agona (4KA32). As shown in Figure 2A, the 3 concentration of WA32 do not show a dose-dependent response: all three concentrations cause a continuous drop in the cell index (i.e. the well impedance as a measure of cell attachment) about 6 hours after compound addition down to 0.2 to 0.3 which is about 20-40% of the relative viability to the control. Similarly, for the treatment of 4KA32, three concentrations including 10^4, 10^5 and 10^6 CFU did not show a dose dependent response: all three concentrations caused a continuous decrease in the cell index down to 0.2 to 0.3 which is about 20-40% of the relative viability to the control (Figure 3A). The CMT-Stylo cells treated with 35µM of doxorubicin showed a gradual decrease in cell index starting from the 34^{th} hour which is 10 hours after compound addition and continue to reduce until 0.1 at the end of the experiment (Figure 2A, 3A). The mean cell index for eight different time interval: 24-30h , 30-36h, 36-42h, 42-48h, 48-54h, 54-60h, 60-66h and 66-72h was obtained for the control, doxorubicin, 3 concentration of wild type *Salmonella* Agona and 3 concentration of genetically modified *Salmonella* Agona (Figure 4B) for statistical analyses using One Way ANOVA. Statistically, all three concentrations of the wild type *Salmonella* Agona (WA32) showed no significant difference between each other ($p>0.05$), besides that, the WA32 also showed no significant difference compared to cells treated with 35µM doxorubicin ($p>0.05$), which is a positive control in this experiment. Similarly for the 4KA32 , all 3 concentrations of treatment showed no significant difference between each other ($p > 0.05$) and to doxorubicin 0.035mM ($p>0.05$). When comparing between WA32 and 4KA32, all three concentrations of wild type *Salmonella* Agona treatment showed no significant difference from each similar to the genetically modified *Salmonella* Agona ($p>0.05$).

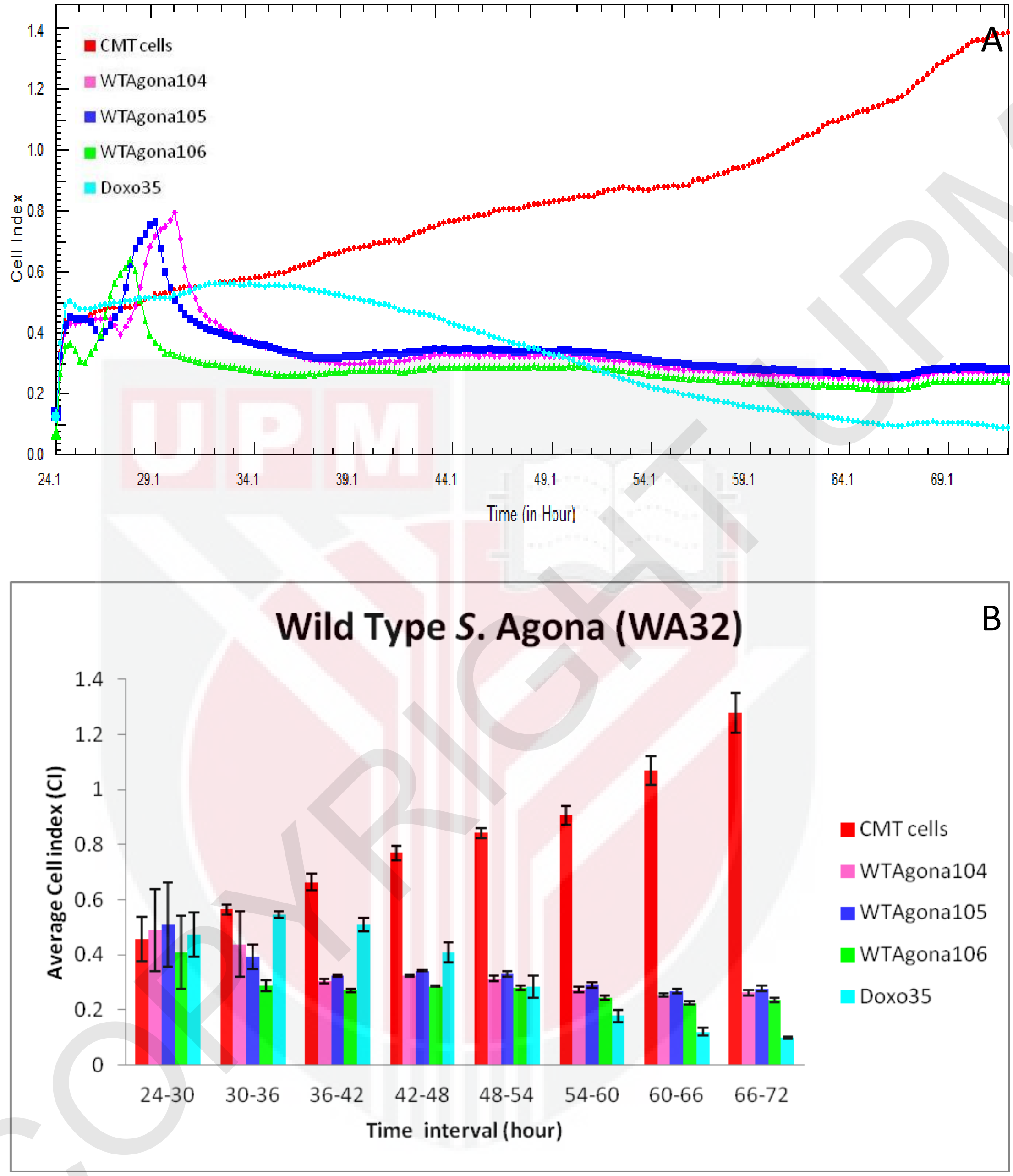

Figure 2. A. Dynamic assessment of cell viability of CMT-Stylo after treated with wild type *Salmonella* Agona (WA32) and 35µM Doxorubicin. (red curve: pure cell with growth media; pink curve: 10^4CFU of WA32; blue curve: 10^5 CFU of WA32; green curve : 10^6 CFU of WA32; light blue curve: 35µM Doxorubicin); **B.** The average cell index of CMT-Stylo treated with WA32, 35µM Doxorubicin and an untreated control at 8 different time interval starting from the 24th hour which is when treatment compound was added.

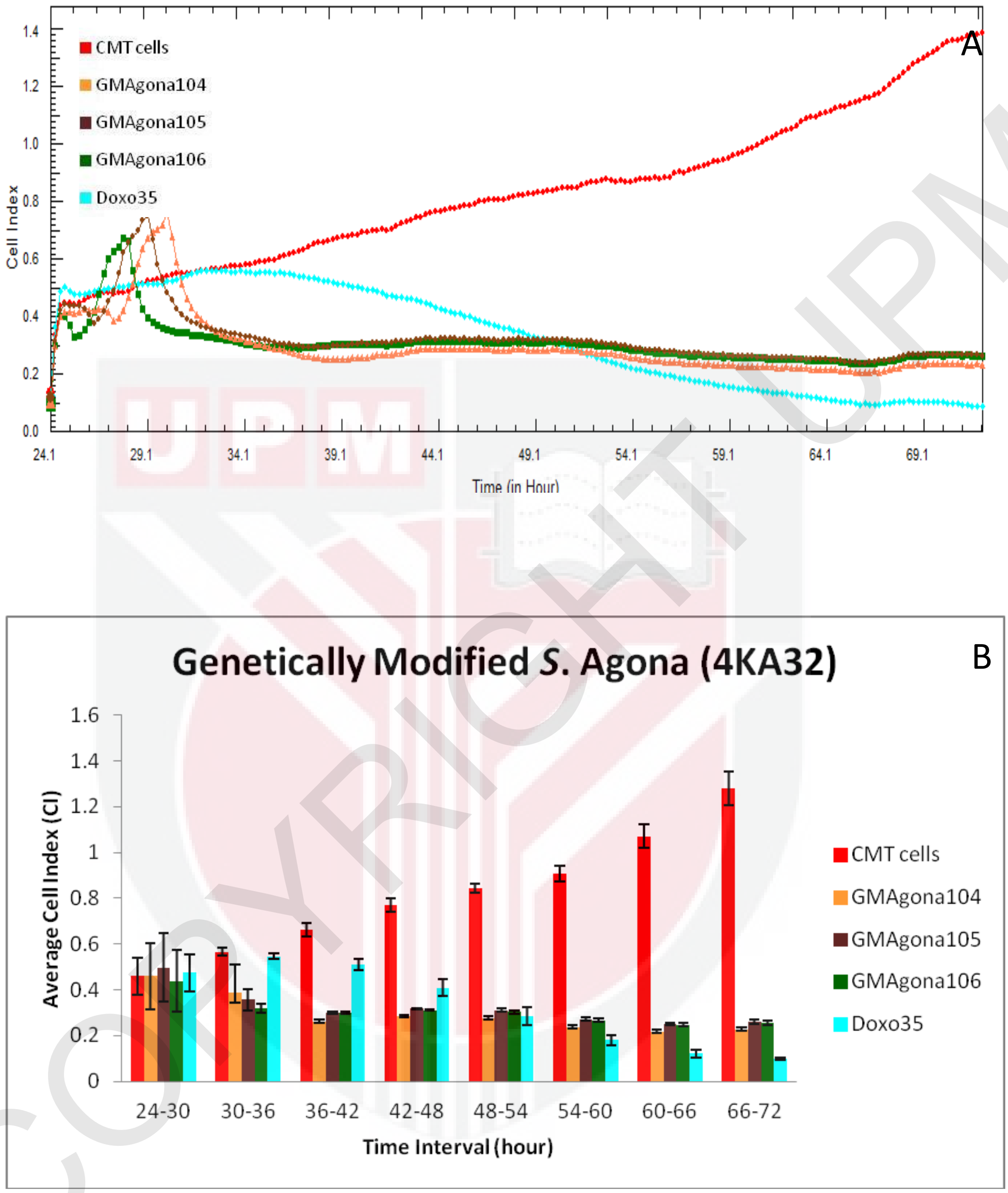

Figure 3. A. Dynamic assessment of cell viability of CMT-Stylo after treated with genetically modified *Salmonella* Agona (4KA32) and 35µM Doxorubicin. (red curve: pure cell with growth media; orange curve: 10^4CFU of 4KA32; brown curve: 10^5 CFU of 4KA32; dark green curve : 10^6 CFU of 4KA32; light blue curve: 35µM Doxorubicin); **B.** The average cell index of CMT-Stylo treated with 4KA32, 35µM Doxorubicin and an untreated control at 8 different time interval starting from the 24th hour which is when treatment compound was added.

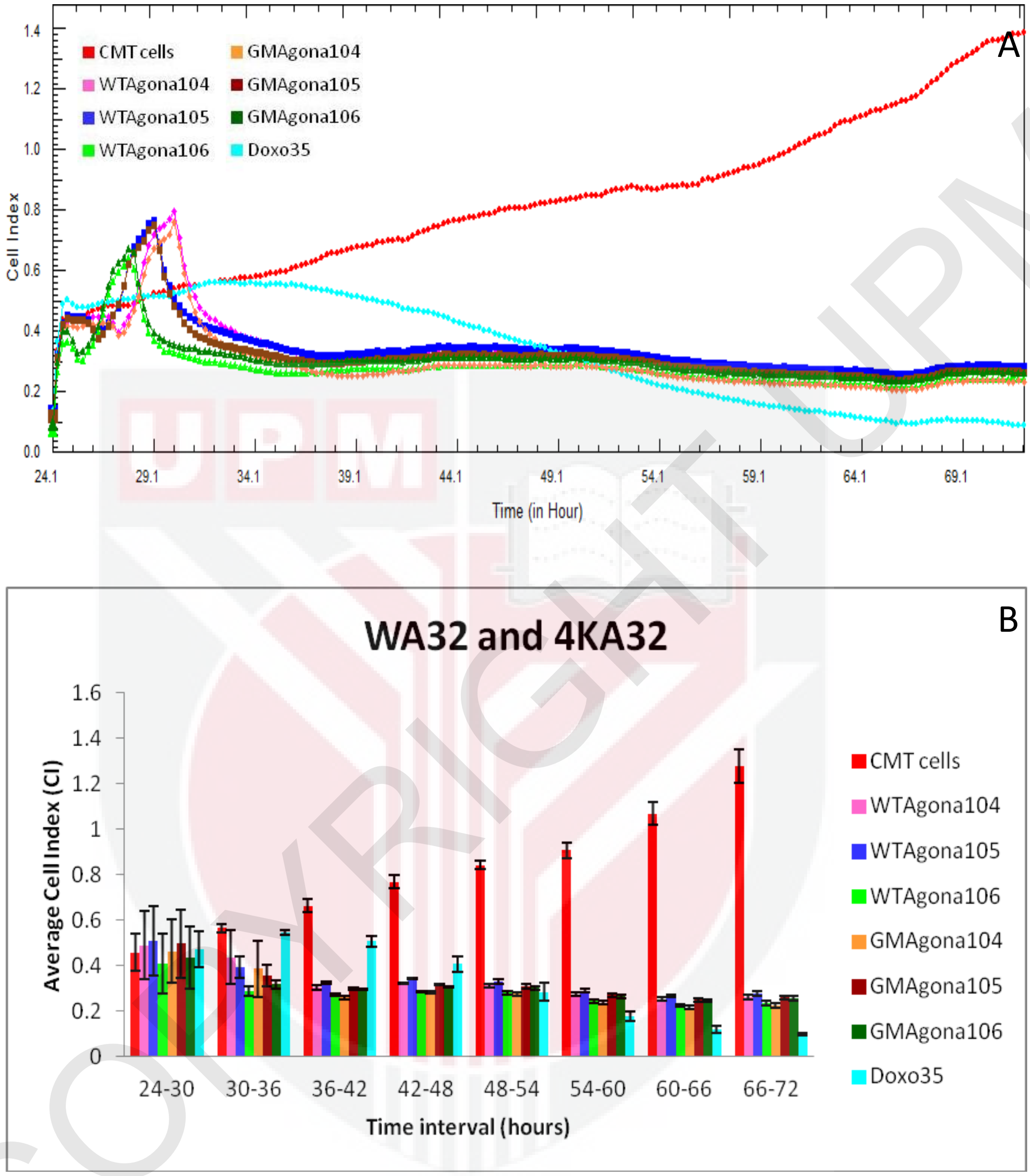

Figure 4. A. Comparison of cell viability of CMT-Stylo treated with Wild type *Salmonella* Agona (WA32), genetically modified *Salmonella* Agona (4KA32) and 35µM Doxorubicin. (red curve: pure cell with growth media; pink curve: 10^4CFU of WA32; blue curve: 10^5 CFU of WA32; green curve : 10^6 CFU of WA32;orange curve: 10^4CFU of 4KA32; brown curve: 10^5 CFU of 4KA32; dark green curve : 10^6 CFU of 4KA32; light blue curve: 35µM Doxorubicin); **B.** Comparison of the average cell index of CMT-Stylo treated with WA32, 4KA32, 35µM Doxorubicin and an untreated control at 8 different time interval starting from the 24th hour which is when treatment compound was added.

4.5 Cell morphology upon treatment

The morphological changes of the CMT-Stylo cells were also observed for each well before and after the treatment procedures. The untreated control well showed cells have grown into 90% confluence with few cells clumping together at 48 hours post treatment. Morphological changes were pronounced in the doxorubicin treated cells, whereby there was cell detachment and rounding of the cells which is most probably attributed by apoptotic cell death. The wells treated with wild type *Salmonella* Agona and genetically modified *Salmonella* Agona showed bacteria overgrowth at the bottom of the well but the canine mammary tumour cells can be seen as clumps of whitish cells in the well suggestive of detachment and apoptotic cell death.

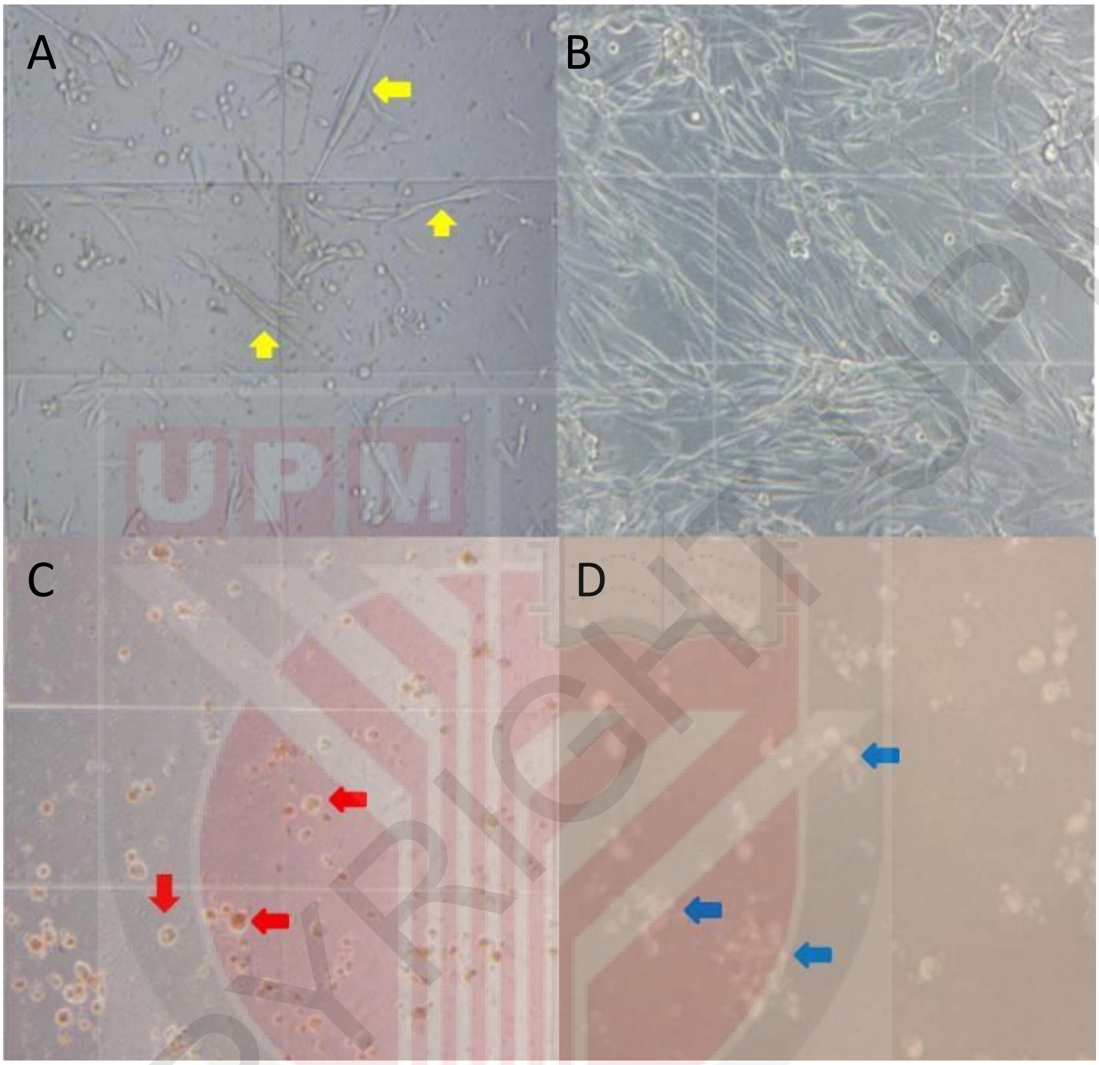

Figure 5. Morphology of cells before and after treatment. **A.** Morphology of cells before treatment. Spindle shape (yellow arrows) shown is the attachment of cells to the bottom of the well. **B**. Negative control well (Media). Cells show 90% confluency 72 hours after seeding. **C.** Positive control well treated with 35.0 µM of doxorubicin. Cells were detached from the bottom and seen as round shaped cells(red arrows). **D.** Bacteria have overgrown the plate surface and rounding of CMT-Stylo cells. Clumps of cells were seen indicating dead cells(blue arrows).

5.0 DISCUSSION

Therapy incorporating anaerobic bacteria for anti-cancer therapeutics provides us with several advantages as compared to other approaches as they can exert anti-tumour effects by preferentially colonize and replicate within the hypoxic tumour environment (Thamm *et al.*, 2005; Pawelek *et al.*, 1997). Studies have been shown over the years that indicate there is an association between bacterial colonization resulting in tumour growth inhibition and subsequently regression in size. Various works has been done on the use of attenuated *Salmonella* Typhimurium in different types of cancer cells but has yet to become a clinical success. Poor performance has been shown by the *Salmonella* strain VNP 20009 in human clinical trials (Leschner and Weiss, 2010). In this study, we evaluated for the first time, the anti-cancer properties of *Salmonella* Agona in a canine mammary tumour cell line grown *in vitro.*

In addition of the CMT-Stylo cell line, primary cell culture from dogs with canine mammary tumour were attempted twice for the establishment of a new cell line. The purpose of establishing a primary cell cultures from tumour tissues is because primary cell cultures mimic closest to the physiological state of cells *in vivo* and generate the relevant data representing the living systems (ATCC, 2012). In this study, both attempts resulted in biological contamination of the cells whereby bacteria were observed within the cell suspension under microscope and colour changes of media. Therefore, the experiment was preceded using the CMT-stylo cell line which is a stable cell line derived from a dog with mammary tumour and established in Malaysia. The possibilities of the biological contaminant entering the culture includes contact with non-sterile supplies, media or solution, particulate or aerosol fallout during culture manipulation, transportation, incubation, contamination of the culture vessels as well as personnel accidents and mistakes. Therefore, ensuring product is sterile and filtered, clean working space, working in a draft free room and reducing the number of personnel would help in reducing the risk of contamination. This can be done by practicing good aseptic techniques, keeping the laboratory clean and routinely monitor contamination.

A preliminary study was done to evaluate the cytotoxic effect comparing the wild type *Salmonella* Typhimurium and wild type *Salmonella* Agona (WA32) on the CMT-stylo cell lines using the RTCA bacterial invasion assay which shown promising results whereby the different concentrations of WA32 were able to exert similar cytotoxic effects as the *Salmonella* Typhimurium on the CMT-Stylo cells. In vitro. This has further reinforced the theory that *Salmonella* Agona is a bacterial candidate with great potential to be used for anti-cancer therapy. Similarly, unpublished data also showed that the attenuated form of the WA32 is able to inhibit the growth of various tumours in murine models *in vivo*, with head and neck cell carcinoma, colorectal cell carcinoma and hepatic cancer from human origins. The *Salmonella* Typhimurium A1-R which has been attenuated through auxotrophy to reduced virulence in mice is able to target tumour cells two to three times more than in the liver because they require purines, pyrimidines and amino acid from tumour cells (Pawelek *et al.*, 1997). The genetically modified *Salmonella* Agona (4KA32) which has its metabolite genes silenced will therefore preferably and selectively adhere, penetrate and grow in the tumour tissues as it will require nutrients resources from tumour tissue for growth.

There were extensive studies done on the use of *Salmonella* Typhimurium on various cancer cells in murine models *in vivo,* and in one particular study, the use of *Salmonella* Typhimurium in dogs with spontaneous neoplasia was conducted in the year 2005 (Thamm *et al.*,2005). In that study, the administration of *Salmonella* VNP20009 became dose limiting as a result of pronounced side effects observed in the dogs in particular pyrexia (Thamm *et al.*, 2005). Since then, there has been no published data on the further investigation of VNP20009 as an anti-cancer agent in dogs *in vivo* which makes our genetically modified *Salmonella* Agona a great candidate to be taken to the next level in *in vivo* studies in dogs. Mutation or attenuation of the bacterium will require the bacteria to target the tumour and kill them effectively after the virulence gene is silenced or removed (Yu *et al.* 2013). The results of the 4KA32 treatment demonstrated reduction of cell index that has no significant difference from the wild type *Salmonella* Agona which makes it to be an effective agent against cancer cells with low pathogenecity. The attenuated form of *Salmonella* is believed to be less toxic compared to the VNP20009 and is as effective as the wild type *Salmonella* Typhimurium.

The bacteria invasion assay performed using the iCELLigence™ RTCA analysis represents a new, quick and label free method to assess the cytotoxic activities of bacterial treatment on cancer cell lines. Variety of techniques has been used over the years for the analysis of host-bacterial interaction, or more specifically cancer-bacterial interaction. Various researchers have adopted techniques like immunofluorescence, electron microscopy and flow cytometry to examine the apoptotic, infected cells that are labelled with dye. Besides that, immunological quantification of mediators is also one of the techniques used for microbial infection of tissues and cells. However, these assays require extensive labelling, washing, lysing and fixation. Furthermore, they analyze the bacterial invasion at a single or few time points after the infection to assess the cellular response and this might lead to incomplete conclusion regarding the cellular process under study (Mou *et al*, 2011). Dynamic investigation using the real-time cell analysis becomes more important because it gives us more information about the cell-bacterial interaction. The iCELLigence^{TM} RTCA system depends on the cell number, morphology and adhesive properties of the cell to yield the read outs in cell index. As shown in Figure 4A-B, the addition of the *Salmonella* Agona resulted in an immediate increased in CI followed by the decline in the CI values. This is hypothesized due to the cancer cells did not regain the normal architecture after the bacterial internalization and therefore resulted in a decrease in CI. Another cell based assay that is commonly used to quantify the cytotoxicity of various drugs on cell proliferation is the 3-4,5-dimethyI(thiazol-2-yl)-3,5-dipherytetradium bromide (MTT) assay. The MTT assay allows the quantification of metabolically active viable cells that reduce the yellow tetrazolium salt to purple blue formazan crystals through the dehydrogenase activity which can be quantified using a spectrophotometer. However, MTT assay is not suitable to be used in this experiment as bacteria such as *Salmonella* has the ability to reduce the MTT salt to formazan crystals and will require the cells to be treated with antibiotics prior to MTT addition (Patel *et al*, 2013). The RTCA technology is able to evaluate multiple physiological changes together with other emerging advances in cell based assay which provides clearer insights into the interaction between bacteria and host (Patel *et al.*, 2013).

Doxorubicin is an anthracycline drug used first extracted from the *Streptomyces peucetius var. caesius* yeast in the 1970. In human, it is routinely used for the treatment of several cancers including breast, lungs, gastric, ovarian, thyroid, lymphoma (Thorn *et al.*, 2011). The mechanism of doxorubicin to act on cancer cell is by intercalation of DNA which leads to inhibition of protein synthesis and free radical formation as well as inhibition of topoisomerase enzyme. It promotes tumour cell apoptosis and is widely used as a broad spectrum chemotherapeutic drug (Yen-Ling *et. al.*, 2014). In this experiment, we can see that there is a gradual drop of the cell index treated with doxorubicin 35µM starting from 34 hours, which is 10 hours post treatment. In comparison to cells treated with *Salmonella*, it has a later onset of action and resulted in lower cell index at the end of the experiment. The dose of doxorubicin was determined based on LD50 dose from previous experiment of testing this cell lines using the MTT proliferation assay (unpublished data, personal communications). The lower cell index observed with doxorubicin effects on CMT-Stylo cell line suggests that it is a good drug to be used to target canine mammary tumour. However, the cytotoxic effects of mammary cancer cell lines due to doxorubicin varies between cell lines and is generally affected by the HSP27 receptor, which an increase in this receptor in the cell can result in resistance to doxorubicin (Yen-Ling *et. al*., 2014). In order to reduce the toxicity of doxorubicin on the other normal cells in the body, genetically modified *Salmonella* will be a good carrier that targets cancer cells and deliver the doxorubicin drug into the specific cancer cells (MacDiarmid *et al.*, 2007). Or genetically modified *Salmonella* can be used solely because it is supposed to be safe to normal cells, targets the cancer cells efficiently and causes regression tumour size.

In this study, both wild type *Salmonella* Agona (WA32) and genetically modified *Salmonella* Agona (4KA32) demonstrate cytotoxic effects on canine mammary tumour cell lines (CMT-Stylo). Cytotoxicity of WA32 and 4KA32 showed similarity and various concentration does not show difference in cytotoxic activity, the attenuated strains is therefore recommended for anti-cancer therapy with minimal pathogenic effect *in vivo*. Future studies can be done using *Salmonella* Agona in *in vivo* studies in dogs with spontaneous neoplasia since the last reported study on *S*. Typhimurium was in 2005. Perhaps we can also further investigate the cellular mechanism of *Salmonella* Agona killing CMT-Stylo cells by labelling it with green fluorescence protein and red fluorescence protein and view under the fluorescence microscopy. Future studies can also be done to evaluate the efficiency of *Salmonella* Agona using it together with other drugs or as vector for drug delivery.

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6.0 CONCLUSION AND RECOMMENDATIONS

Genetically modified bacteria have offered new perspective to effectively treat mammary gland tumour as well as in several other types of tumours in humans. Determining the most effective strain of genetically modified bacteria that can effectively invade and target tumour tissues and destroy the cancer cells without causing side effect to normal cells is still something under pursuit of every researcher. In this study, for the first time, it has been demonstrated that the wild type and genetically modified and attenuated (for selected metabolite and virulence genes) *Salmonella* Agona has anti-cancer effects in canine mammary gland tumour cells *in vitro*. The mechanism of *Salmonella* Agona in causing the regression in the cell index is still unknown; therefore, future studies should be done in this grey area. Therefore, further *in vivo* studies in murine and canine models with spontaneous neoplasia are warranted before this sort of therapeutic strategy is brought to the clinics.

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APPENDIX

Appendix A

Multiple comparisons of cell index between various treatment

Multiple Comparisons

Dependent Variable: Cell_index

Tukey HSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
CMT only	WT Agona 104	.5145*	.0662	.0000	.3060	.7229
	WT Agona 105	.4773*	.0662	.0000	.2688	.6857
	WT Agona 106	.4611*	.0662	.0000	.2526	.6695
	Doxorubicin	.4919*	.0662	.0000	.2835	.7004
	GM Agona 104	.5465*	.0662	.0000	.3380	.7549
	GM Agona 105	.4885*	.0662	.0000	.2800	.6969
	GM Agona 106	.4674*	.0662	.0000	.2589	.6758
WT Agona 104	CMT only	-.5145*	.0662	.0000	-.7229	-.3060
	WT Agona 105	-.0372	.0662	.9992	-.2456	.1713
	WT Agona 106	-.0534	.0662	.9921	-.2619	.1551
	Doxorubicin	-.0225	.0662	1.0000	-.2310	.1859
	GM Agona 104	.0320	.0662	.9997	-.1764	.2405
	GM Agona 105	-.0260	.0662	.9999	-.2344	.1825
	GM Agona 106	-.0471	.0662	.9963	-.2556	.1614
WT Agona 105	CMT only	-.4773*	.0662	.0000	-.6857	-.2688
	WT Agona 104	.0372	.0662	.9992	-.1713	.2456
	WT Agona 106	-.0162	.0662	1.0000	-.2247	.1922
	Doxorubicin	.0146	.0662	1.0000	-.1938	.2231
	GM Agona 104	.0692	.0662	.9651	-.1393	.2777
	GM Agona 105	.0112	.0662	1.0000	-.1973	.2197
	GM Agona 106	-.0099	.0662	1.0000	-.2184	.1985
WT Agona 106	CMT only	-.4611*	.0662	.0000	-.6695	-.2526
	WT Agona 104	.0534	.0662	.9921	-.1551	.2619
	WT Agona 105	.0162	.0662	1.0000	-.1922	.2247
	Doxorubicin	.0309	.0662	.9998	-.1776	.2393
	GM Agona 104	.0854	.0662	.8987	-.1230	.2939
	GM Agona 105	.0274	.0662	.9999	-.1810	.2359
	GM Agona 106	.0063	.0662	1.0000	-.2021	.2148
Doxorubicin	CMT only	-.4919*	.0662	.0000	-.7004	-.2835
	WT Agona 104	.0225	.0662	1.0000	-.1859	.2310
	WT Agona 105	-.0146	.0662	1.0000	-.2231	.1938
	WT Agona 106	-.0309	.0662	.9998	-.2393	.1776
	GM Agona 104	.0546	.0662	.9910	-.1539	.2630
	GM Agona 105	-.0034	.0662	1.0000	-.2119	.2050
	GM Agona 106	-.0246	.0662	.9999	-.2330	.1839

GM Agona 104	CMT only	-.5465*	.0662	.0000	-.7549	-.3380
	WT Agona 104	-.0320	.0662	.9997	-.2405	.1764
	WT Agona 105	-.0692	.0662	.9651	-.2777	.1393
	WT Agona 106	-.0854	.0662	.8987	-.2939	.1230
	Doxorubicin	-.0546	.0662	.9910	-.2630	.1539
	GM Agona 105	-.0580	.0662	.9871	-.2665	.1505
	GM Agona 106	-.0791	.0662	.9301	-.2876	.1293
GM Agona 105	CMT only	-.4885*	.0662	.0000	-.6969	-.2800
	WT Agona 104	.0260	.0662	.9999	-.1825	.2344
	WT Agona 105	-.0112	.0662	1.0000	-.2197	.1973
	WT Agona 106	-.0274	.0662	.9999	-.2359	.1810
	Doxorubicin	.0034	.0662	1.0000	-.2050	.2119
	GM Agona 104	.0580	.0662	.9871	-.1505	.2665
	GM Agona 106	-.0211	.0662	1.0000	-.2296	.1874
GM Agona 106	CMT only	-.4674*	.0662	.0000	-.6758	-.2589
	WT Agona 104	.0471	.0662	.9963	-.1614	.2556
	WT Agona 105	.0099	.0662	1.0000	-.1985	.2184
	WT Agona 106	-.0063	.0662	1.0000	-.2148	.2021
	Doxorubicin	.0246	.0662	.9999	-.1839	.2330
	GM Agona 104	.0791	.0662	.9301	-.1293	.2876
	GM Agona 105	.0211	.0662	1.0000	-.1874	.2296

*. The mean difference is significant at the 0.05 level.