ORIGINAL ARTICLE

Tumour Growth Inhibition and Systemic Responses of Δ*sopB*Δ*sopD*Δ*pipD* Disrupted *Salmonella* Agona and *Salmonella* Typhimurium in Mice

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ABSTRACT

Introduction: Bacteria had long been known to have tumour-targeting and tumour inhibition capabilities and have re-emerged into the limelight of cancer research as a possible alternative treatment for solid tumours. Conventional therapies for solid tumours are either by surgery, chemotherapy, radiotherapy, which are very invasive and non-specific to the tumours and results in various adverse effects on the patients. Bacterial Mediated Tumour Therapy often utilises attenuated bacteria as therapeutic agents to ensure reduced pathogenicity of the strains. However, this often results in lower invasiveness towards the tumours itself. In this study, we studied the tumour inhibition capabilities of Salmonella Pathogenicity Island (SPI) attenuated *Salmonella* Typhimurium (*S.* Typhimurium) and *Salmonella* Agona (*S.* Agona), specifically with attenuation of *sopB, sopD*, and *pipD* genes. **Methods:** Balb/c mice bearing CT26 tumours were inoculated with *S.* Typhimurium and *S.* Agona, both unattenuated and Δ*sopB*Δ*sopD*Δ*pipD* attenuated strains. Tumour volumes were monitored daily. Organs and blood were collected for plasma liver enzyme analysis and histopathology studies on testis, liver, kidneys and brain. **Results:** The Δ*sopB*Δ*sopD*Δ*pipD S.* Agona treated group showed improved inhibition of tumour growth with 51.11% tumour volume reduction compared to unattenuated *S.* Agona. The Δ*sopB*Δ*sopD*Δ*pipD* strains have also shown lesser systemic effects as observed in plasma and histopathological studies) compared to its unattenuated counterparts. **Conclusion:** The present study showed that Δ*sopB*Δ*sopD*Δ*pipD S.* Agona has a great potential to be utilised as tumour therapeutic agent as it exerts lesser systemic effect while having similar tumour inhibition capabilities as the well-studied *S.* Typhimurium strain.

Keywords: Bacterial Mediated Tumour Therapy, Tumour Inhibition, Tumour-targeting, Salmonella Pathogenicity Island, *Salmonella* Agona

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INTRODUCTION

Conventional tumour therapies offered as interventions for tumour patients include surgery, radiotherapy and chemotherapy, which comes with its own set of limitations. Surgery and radiotherapy are only successful in eradication of localised tumours and are very invasive, whereas chemotherapy is not specific to tumour cells and causes severe side effects by effecting normal and healthy cells too (1). Chemotherapeutics agents often are only effective in primary tumours and are less sensitive to metastatic tumours and had been associated with adverse effects to patients such as the development of myocardial ischemia and cardiotoxicity (2,3). These limitations urge the need for new tumour interventions. Bacterial Mediated Tumour Therapy (BMTT) is a branch of therapy which falls under the category of cancer immunotherapy and is gaining interest in cancer research (4). Immunotherapy utilises the patient's immune system to attack the malignant tumours after it had been stimulated or enhanced. The first systemic study of immunotherapy for the treatment of malignant tumour was developed in the year 1981 by William B. Coley. He observed that a patient with an inoperable malignant tumour showed regression in the tumour size after he developed erysipelas, a bacterial skin infection that is caused by Group A *Streptococci* (5). Species such as *Streptococcus, Clostridium, Bifidobacterium, Salmonella, Escherichia coli and Listeria* are being studied extensively in this area since had been shown to accumulate and proliferate in solid tumours (6). *Salmonella typhimurium* (*S. Typhimurium*) is the bacterial species that is mostly studied in the area of BMTT, and the VNP 20009 is the only *Salmonella* strain evaluated in phase I clinical study for the treatment

of nonresponsive metastatic melanoma or renal cell carcinoma (7).

However, there is some concern regarding BMTT. The properties that allow microorganisms such as *Salmonella* to invade tumour cells could also cause invasion to other major organs and cause infection to the patients, especially to immunosuppressed patients (8). The ideal tumour-targeting bacterial strain would be bacteria with reduced virulence towards the host and could colonise tumours specifically over other tissues. One strategy to overcome this hurdle is by developing and studying bacterial strains with different combinations of attenuated genes, which may allow for the discovery of an attenuated strain which may specifically invade the tumour cells while having reduced pathogenicity towards the patients (9). The importance of this balance is seen in the VNP20009 strain, which showed promising results in in vivo studies, however, the phase I clinical study reported poor outcome, and it was attributed to the inability of the attenuated strain to target and colonise tumours as it was cleared rapidly in human patients (10). Besides attenuation, using a less pathogenic strain might also solve this problem. *Salmonella* Agona (*S.* Agona) used in this study was isolated from a vegetable that is usually consumed raw just like a salad, by the Malay ethnic and is locally known as 'ulam'. Taking into consideration of the rarity of an outbreak related to *S.* Agona, even when it is commonly isolated from raw vegetables, this suggests the possibility that its pathogenicity is lower when compared to *S.* Typhimurium (11,12). We also investigated the effects of attenuation of the *sopB*, *sopD*, and *pipD* genes of both strains, the *S.* Typhimurium and *S.* Agona on tumour-bearing mice. These genes are confined to the *Salmonella* Pathogenicity Island (SPI), which encodes many of the virulence phenotypes of the *Salmonella* species, for example, the host-cell invasion and intracellular pathogenesis (13). The *sopB* and *sopD* genes are responsible for the expression of *sopB* and *sopD* which are part of Type III Secretion System 1 (T3SS1) effector proteins and contributes to intestinal inflammation in bovine ligated ileal loops and streptomycin-pretreated mice following *Salmonella* inoculation (14). The *pipD* gene is part of SPI-5 enteritis- associated genes which are mainly associated with enteropathogenesis (15,16). The attenuation of these genes are hypothesised to further reduce the pathogenicity of the bacterial strains towards the subjects.

This study aims to investigate the potential of *S.* Agona as tumour therapy agent and its pathogenicity compared to *S.* Typhimurium, that had been extensively studied for its antitumour capacity (17). There are limited studies that show the tumour inhibition capacity of *S.* Agona.

Besides looking into tumour inhibition capabilities of the bacterial strains and improvement of survival of the CT26 tumour-burdened Balb/c mice, the effect of the bacterial strains on major organs such as liver, kidney, brain and testis are also studied. The CT26 xenograft model is a well-established model for tumour study and is often used in studies looking on antitumour effects of BMTT agents (18–21). Evaluations on these organs are done by observing the histopathological changes to the tissues of these organs and by observing the level of hormones related to these organ systems.

MATERIALS AND METHODS

Bacterial strains and maintenance

S. Typhimurium (ATCC 14028) was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) while *S.* Agona was isolated from indigenous vegetable which is often consumed raw (22). The Δ*sopB*Δ*sopD*Δ*pipD* attenuated *S.* Typhimurium and *S.* Agona were developed in previous work using the Targetron Gene Knockout system (Sigma-Aldrich, Germany), which is a site-specific group II intron knockout approach and were stored in 15 % glycerol at -20°C (12). Besides detailing the protocol for the attenuation, Khoo et al. (2015) also described protocols to verify the insertion of introns in the strains. The bacteria strains were thawed, and later overnight cultures were prepared (37°C and 200 rpm) in Luria-Bertani (LB) media (Merck KGaA, Germany). The cultures were later appropriately diluted in phosphate-buffered saline (PBS).

Cell Line and maintenance

Mouse (*Mus musculus*) colon carcinoma cell line, CT26 cells were generously provided by the Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia. The CT26 cells were maintained at 37 °C in a humidified incubator with 5% Carbon Dioxide in Dulbecco's Modified Eagle Medium (DMEM) media (Merck Millipore, Germany) supplemented with Fetal Bovine Serum (Sigma-Aldrich, Darmstadt, Germany), penicillin 100 U/ml and Streptomycin 100µg/ml. Monolayer cell suspension of the cells was prepared using 0.25% trypsin-EDTA solution, which detaches the cells from the flasks.

In vivo xenograft murine model of colon carcinoma

Balb/c mice of six weeks old, both female and male were used in this study. All animal experiments were performed according to procedures that had been proposed to and approved by the Animal House and Use Committee of the Faculty of Medicine and Health Sciences of Universiti Putra Malaysia (UPM/FPSK/PADS/BR-UUH/00434). After a week of the adaptation period, $3x10^6$ CT26 cells in 100 µl PBS were injected subcutaneously on the left flank of the mice. The tumours were allowed to grow for three weeks before treatments were injected into the mice.

Tumour inhibition studies

10^3 CFU/100 µl PBS of unattenuated *S.* Typhimurium, Δ*sopB*Δ*sopD*Δ*pipD* *S.* Typhimurium, unattenuated

S. Agona and Δ*sopB*Δ*sopD*Δ*pipD S.* Agona were inoculated intratumourally (IT) (23). The sizes of the tumours were measured using a digital calliper daily for a maximum of 20 days or until the tumour volume reached 2000mm^3 (24). The tumour volumes were then calculated using the formula $V=\pi/6xf(lengthxwidth)^{3/2}$, where 'V' represents the tumour volume (mm^3), while the value of 'f' is 1.58 ± 0.01 for females and 1.69 ± 0.03 for males (25). Relative tumour volume was calculated comparing the tumour volume on each time point to the initial tumour volume on day 1 (26).

Blood plasma analysis

Blood was collected from each mouse via cardiac puncture method (27). For white blood cell count, the blood samples were analysed using Sysmex KX-21 Automated Haematology Analyser (Sysmex Corporation, Japan). For the differential white blood count, a blood film was prepared on a glass slide, and it was then stained using Leishman's stain. Different types of cells were identified and counted. The testosterone levels in the plasma were analysed using TESTO-CTK radioimmunoassay kit (DiaSorin, Italy) and processed by COBRA II auto-gamma analyser (Packard, UK). Levels of aminotransferases, specifically alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were also analysed using Hitachi 902 automated analyser (Roche, Germany).

Histopathological assessment

Organs such as testes, brain, liver and kidneys were fixed in 10% formalin immediately after the organs were removed from the mice. The organs and tissue were then processed, sectioned and then stained using Haematoxylin and Eosin (H&E) stain. The organs were then observed using a light microscope for any histological changes in the organs.

Statistical analysis

One-way ANOVA with Tukey's post-hoc test was performed using GraphPad Prism version 8.0.2 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com. The results of this study are presented as mean ± standard deviation (SD), and the differences were statistically significant at probability values of less than alpha 0.05 ($p < 0.05$). Significant results were then further analysed and compared using Dunnett's multiple comparisons test.

RESULTS

Effects of treatments on the changes of tumour volumes and survival

Relative tumour volumes were calculated by comparing tumour volumes on each timepoint to initial tumour volumes on day 1 (Table I). The relative tumour growth curve shows the difference in the growth of tumour at different time points in relation to its initial volume until the endpoint (20 days post-treatments or when the tumour volume reached 2000mm^3) (Figure 1A). All bacterial treatments showed an improved inhibition of the tumour growth when compared to the control-treated group; however, no significant differences were seen between the different bacterial treatments for the relative tumour volume on day 20 (Figure 1B).

Table I: Mean of relative tumour volumes at different time points for each group

Days	*S.* Agona	Δ*sopB*Δ*sopD*Δ*pipD S.* Agona	*S.* Typhimurium	Δ*sopB*Δ*sopD*Δ*pipD S.* Typhimurium	PBS
1	1.00	1.00	1.00	1.00	1.00
3	1.47	1.53	0.69	1.77	1.69
6	2.36	1.55	1.46	2.17	3.88
9	3.78	2.44	1.92	3.49	7.70
12	4.32	2.25	1.65	5.57	10.97
15	7.73	3.14	2.12	10.51	NA
18	10.06	4.54	3.93	11.06	NA
20	10.37	5.30	4.58	10.51	NA

NA – Data not available since animals were sacrificed because tumour volume exceeded 2000mm^3.

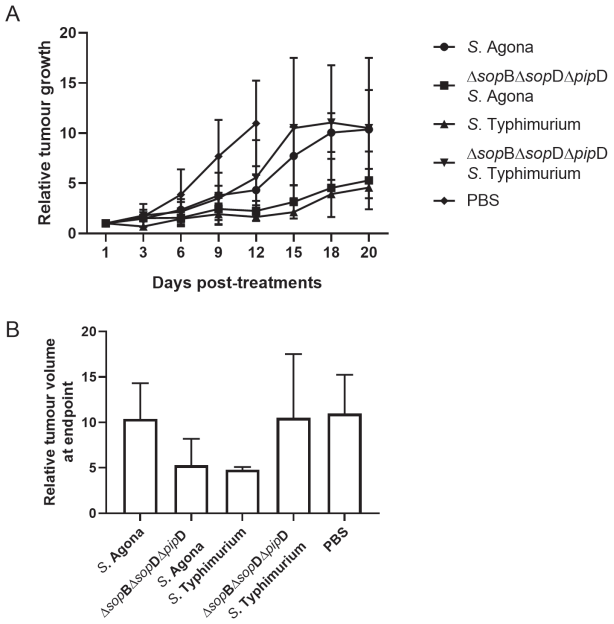

Figure 1: Effects of bacterial treatments on the relative tumour volume observed post-inoculation of the treatments for tumour-bearing BALB/c mice. The relative tumour growth compared to the initial tumour volume were analysed for a period of 20 days post-treatment (A). The relative tumour volumes at day 20 were compared between the treatment groups ($p \leq 0.05$). Data are displayed as mean ± SD (n = 4) (B).

Control-treated tumour-burden mice showed mean relative tumour growth of 10 times of its initial volume on day 12, after which the mice did not survive. The largest inhibition of relative tumour volumes was shown by subjects treated with the unattenuated *S.* Typhimurium with mean relative tumour volume of 4.79, followed by subjects treated with Δ*sopB*Δ*sopD*Δ*pipD S.* Agona (5.30) and unattenuated *S.* Agona (10.37). The least inhibition with the mean relative tumour volume of 10.51 was showed by subjects treated with Δ*sopB*Δ*sopD*Δ*pipD S.*

Typhimurium.

There were no significant differences seen in the inhibition and survival effects of the treatments in relation to the gender of the tumour-burdened mice treated (Data not shown).

Effects of different treatments on the expression of plasma testosterone, aspartate aminotransferase, alanine aminotransferase and blood neutrophil count

The blood was collected from the subjects 20 days post-inoculation, and the plasma was used for the analysis of plasma testosterone, AST and ALT levels and neutrophil blood count.

As shown in Figure 2, treating the tumour-bearing mice with different strains of bacteria showed a significant reduction in plasma testosterone level, except for treatment with Δ*sopB*Δ*sopD*Δ*pipD S.* Agona (0.30 ng/mL), which shows insignificant difference with the control group treated mice (0.36 ng/mL). *S.* Agona, regardless of the unattenuated or attenuated strains, showed the least reduction of the plasma testosterone level when compared to *S.* Typhimurium. The *ΔsopBΔsopDΔpipD S.* Agona and Δ*sopB*Δ*sopD*Δ*pipD S.* Typhimurium (0.16 ng/mL) strains both showed a lesser reduction in plasma testosterone level when compared to the unattenuated strains of *S.* Agona (0.15 ng/mL) and *S.* Typhimurium (0.04 ng/mL).

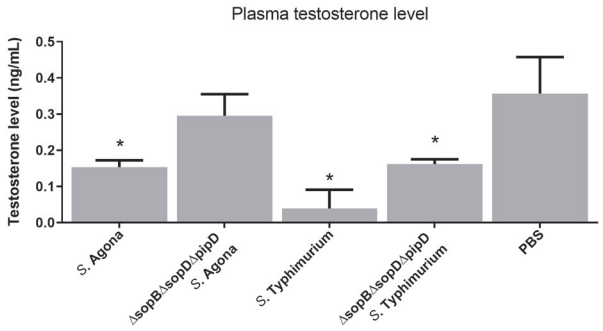

Figure 2: Effects of bacterial treatments on plasma testosterone levels of treated tumour-bearing BALB/c mice. The plasma testosterone level analysis post-treatments showed a reduction for all bacterial treated tumour-burdened mice when compared to the control (PBS treated) group (p≤0.05). *ΔsopBΔsopDΔpipD S.* Agona treated tumour-bearing mice showed the least and insignificant reduction. Data are displayed as mean ± SD (n = 5).

A significant increase was observed in the plasma ALT, AST (Figure 3) and neutrophil levels (Figure 4) for all the treatments when compared to control subjects. It was observable that *S.* Agona treated mice showed a lesser increase in the parameters compared to *S.* Typhimurium treated mice. The attenuated strains show a lesser increase in the parameters when compared to their unattenuated counterparts.

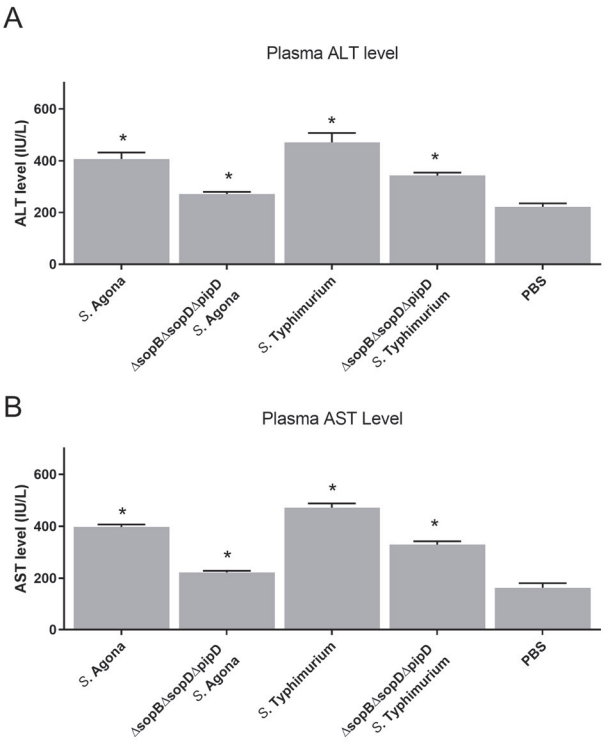

Figure 3: Effects of bacterial treatments on plasma liver enzymes levels of treated tumour-bearing BALB/c mice. The analysis of plasma liver enzymes, both ALT levels (A) and AST levels (B) showed significant increase for all bacterial treated tumour-burdened mice when compared to the control (PBS treated) group (p≤0.05). *ΔsopBΔsopDΔpipD S.* Agona treated tumour-bearing mice showed the least increase in plasma liver enzymes level. Data are displayed as mean ± SD (n = 4).

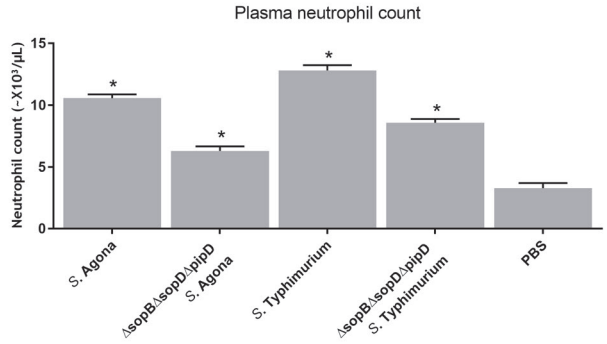

Figure 4: Effects of bacterial treatments on plasma neutrophils levels of treated tumour-bearing BALB/c mice. The analysis of plasma neutrophil count shows significance increases for all bacterial treated tumour-burdened mice when compared to the control (PBS treated) group (p≤0.05). *ΔsopBΔsopDΔpipD S.* Agona treated tumour-bearing mice showed the least increase in plasma neutrophil count. Data are displayed as mean ± SD (n = 4).

Histopathological assessment

H&E staining of the seminiferous tubules sections of the tumour-bearing mouse treated with all of the bacterial treatments showed normal, well-defined histological structures with the absence of inflammation and necrosis

as compared to the control group. The presence of inflammatory cells, such as neutrophils was also not observed (Figure 5A-E).

Histopathology of the liver shows that the parenchyma of the liver for control-treated mice is well preserved (Figure 5F), while necrosis is observable in the parenchyma of mice treated with unattenuated and Δ*sopB*Δ*sopD*Δ*pipD S.* Typhimurium (Figure 5I-J) and unattenuated *S.* Agona (Figure 5G). Sinusoid dilation is observable in the parenchyma of the liver of mice treated with unattenuated *S.* Typhimurium and *S.* Agona. Kupffer cells are observable in the parenchyma of the liver of mice treated with Δ*sopB*Δ*sopD*Δ*pipD S.* Agona (Figure 5H).

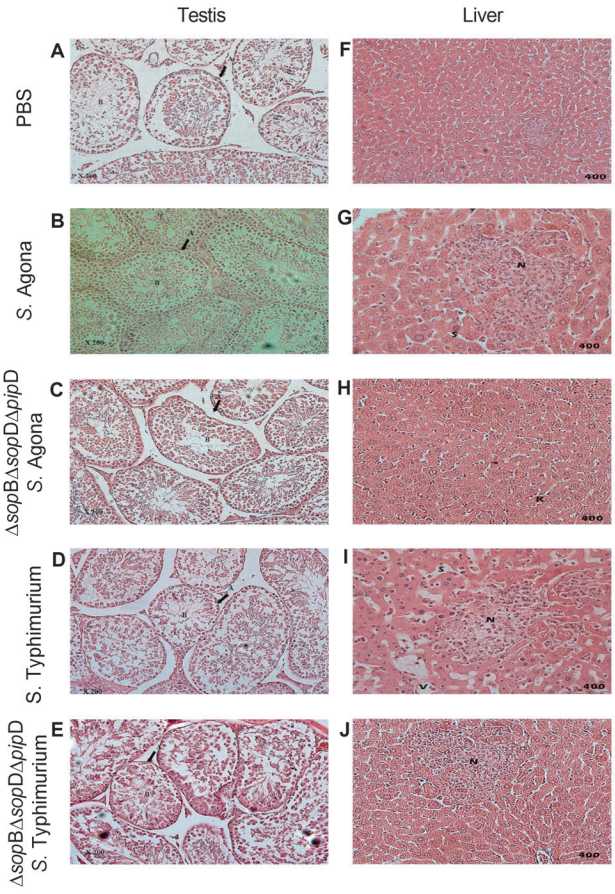

Figure 5: Haematoxylin and eosin (H&E) staining of testis and liver. A-E: Representative of histopathology analysis of seminiferous tubules of testis sections (x200). A, seminiferous epithelium; B, interstitial tissue. F-J: Representative of histopathology analysis of liver sections (x400). N, necrosis; S, sinusoid dilation; K, Kupffer cells; V, vacuolisation of hepatocytes.

Presence of glomeruli, distal tubules and proximal tubules were observed in the cortex of control-treated tumour-bearing mice (Figure 6A). Congested glomeruli is observable in the cortex of tumour-bearing mice treated with both unattenuated and Δ*sopB*Δ*sopD*Δ*pipD S.* Typhimurium (Figure 6D-E) and the unattenuated *S.* Agona (Figure 6B). Tubular destruction is also observable

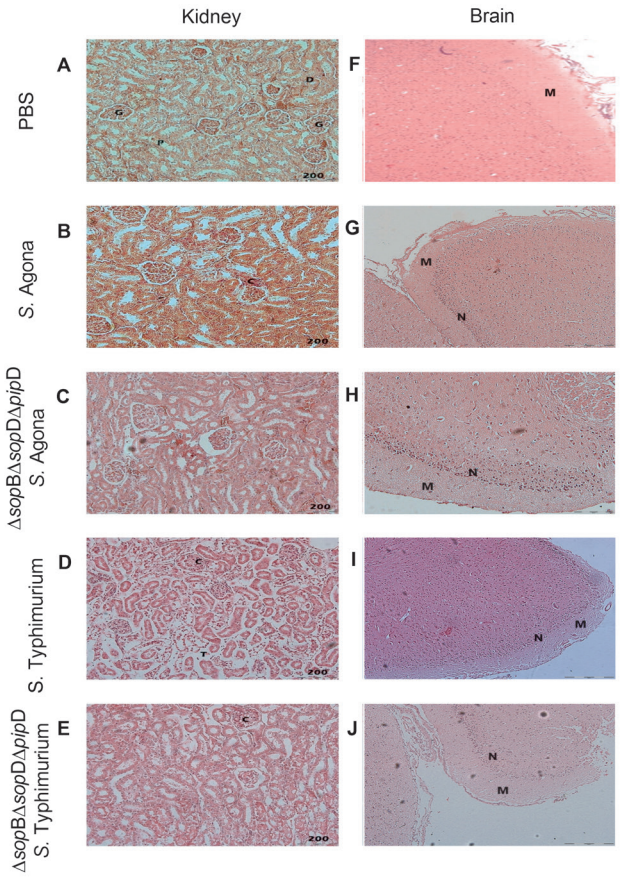

Figure 6: Haematoxylin and eosin (H&E) staining of kidney and brain. A-E: Representative of histopathology analysis of kidney sections (x400). G, glomeruli; D, distal tubules; P, proximal tubules; C, congested glomeruli; T, tubular destruction. F-J: Representative of histopathology analysis of brain sections (x100). N, neutrophils; M, meninges.

in the cortex of mice treated with unattenuated. *S.* Typhimurium. For tumour-bearing mice receiving Δ*sopB*Δ*sopD*Δ*pipD S.* Agona (Figure 6C) treatment, the cortex is covered by the presence of polymorphonuclear cell.

Neutrophils are observable in the meninges of tumour-bearing mice that received both unattenuated and Δ*sopB*Δ*sopD*Δ*pipD* of both strains of *Salmonella* (Figure 6G-J) and is not present in mice treated with control (Figure 6F). However, no signs of inflammation or necrosis were observable in each group.

DISCUSSION

A major problem faced by researchers in the field of BMTT is to develop a bacterial strain that has an ideal balance between its invasiveness for therapeutic benefits and its pathogenicity towards the subject receiving it (4). Over-attenuation of bacterial strains after deletion of a particular gene or after passaging of the strains (in vivo or in vitro) might reduce the therapeutic efficacy of the developed bacterial strains (28,29). Taking these important points into consideration, the invasiveness towards the tumours and the pathogenicity

of the bacterial strains, both directly portrayed by an improvement of the survival and the systemic effects exerted by the strains to the tumour-bearing mice are investigated in this study.

S. Agona (unattenuated and attenuated) treated mice showed improvement in its survival when compared to control while still offers similar tumour inhibition capabilities as *S.* Typhimurium. The attenuation of Δ*sopB*Δ*sopD*Δ*pipD* genes showed improvement in tumour inhibition for the *S.* Agona strain. Δ*sopB* mutation in *S.* Typhimurium is shown to enhance the T-helper type 2 (Th2) response, which is part of adaptive immune cells (30). *sopB* proteins protect *Salmonella* -infected cells from undergoing apoptosis; therefore, the Δ*sopB* mutant infected cells no longer have the apoptosis pathway inhibited, which improves antigen uptake by DC and improves presentation to the immune system (31). It was observed in a study that the Δ*sopB* and Δ*sopD* double mutant *S.* Agona did not contribute to improvement in tumour suppression compared to its parental strain (32). It was however observed from this study that addition of Δ*pipD* gene with a Δ*sopB* and Δ*sopD* double mutant *S.* Agona did improve the tumour inhibition capabilities compared to the unattenuated *S.* Agona strain. Several studies have shown the connection between the tumour inhibition and the survival of the tumour-bearing mice, where tumour inhibition was shown to improve the survival of the treated mice (33–35). This was observable from Figure 1(A), where control-treated mice reached its maximum relative tumour growth, after which the subjects did not survive. The bacterial-treated mice showed increase survival as it offered better inhibition of tumours.

Both *Salmonella* strains showed low pathogenicity towards the treated tumour-bearing mice. It was also observed that the attenuation of the Δ*sopB*Δ*sopD*Δ*pipD* genes did not show significant improvement in the survival of the treated mice when compared to the unattenuated strains. This finding resonates with the results that we had obtained in the previous study. It was observed that the attenuation of Δ*sopB*Δ*sopD*Δ*pipD* genes in both serovars showed no significant decrease in the virulence of the strains in C. elegans (12).

S. Agona showed lesser suppressive effects on the plasma testosterone level when compared to *S.* Typhimurium. Δ*sopB*Δ*sopD*Δ*pipD* strains, both for *S.* Agona and *S.* Typhimurium were shown to exert a lesser suppressive effect on the plasma testosterone level. In a condition where the immune system is being activated, either by live pathogens or non-pathogenic antigens, it is shown to have a strong suppressive effect on the testosterone levels (36). This suppressive effect on the testosterone level is observable in Figure 2, where all bacterial treatment showed a significant reduction in the plasma testosterone level of the tumour-bearing mice receiving it.

The histopathology of the seminiferous tubules of testes shows no abnormal structures, inflammation, necrosis and presence of any inflammatory cells. This indicates that the suppression of testosterone is not due to direct damage to the testis, but it may be affecting the synthesis of testosterone by steroid-producing cells such as the Leydig cells. It has been shown that cytokines play an essential role in mediating differential effects on steroid-producing cells especially in the condition of acute stress, such as during an infection or inflammation, which results in the activation of the adrenal while inhibiting the process of gonadal steroidogenesis (37).

Tumour-bearing mice from all treatment groups showed a similar pattern of a significant increase in the plasma liver enzymes (ALT and AST) when compared to the control-treated group. *S.* Typhimurium showed the highest plasma liver enzymes level, followed by *S.* Agona. The Δ*sopB*Δ*sopD*Δ*pipD* strains showed lesser changes to the liver enzymes level. From all the four bacterial treatments, Δ*sopB*Δ*sopD*Δ*pipD S.* Agona showed the least increase in the liver enzymes level. Aminotransferase, both ALT and AST are liver enzymes that are sensitive and reflects liver injury. Increasing levels of AST and ALT in the serum is associated with hepatocellular damage, necrosis and apoptosis in liver tissue (38,39). It is observed that the Δ*sopB*Δ*sopD*Δ*pipD S.* Agona showed the least increase in liver enzyme levels compared to its parental strain and *S.* Typhimurium, suggesting it induces fewer effects on the liver. These findings are supported by the histopathology findings.

In this study, from the histopathology analysis of the liver, necrosis are observable for tumour-bearing mice receiving unattenuated *S.* Typhimurium, unattenuated *S.* Agona and Δ*sopB*Δ*sopD*Δ*pipD S.* Typhimurium treatments. Sinusoid dilation on the parenchyma of the liver is observable for the liver of tumour-bearing mice receiving unattenuated *S.* Typhimurium and unattenuated *S.* Agona treatments. Kupffer cells were observable in the parenchyma of the liver for Δ*sopB*Δ*sopD*Δ*pipD S.* Agona treated tumour-bearing mice. These findings are parallel to the findings for the plasma aminotransferase levels discussed above. Higher levels of plasma aminotransferase are observed with more damage observed in the histopathology analysis of the liver.

Inflammations of the kidneys of the tumour-bearing mice receiving the bacterial treatments were observable in varying degrees from the histopathological analysis that were carried out. Congested glomeruli and tubular destruction were observable in the cortex of mice treated with unattenuated *S.* Typhimurium. Congested glomeruli were also observable in mice treated with unattenuated *S.* Agona and Δ*sopB*Δ*sopD*Δ*pipD S.* Typhimurium. The cortex of Δ*sopB*Δ*sopD*Δ*pipD S.* Agona treated tumour-bearing mice kidneys were shown to be covered with the presence of polymorphonuclear cells. As what was

observed from the histopathological analysis of the liver, Δ*sopB*Δ*sopD*Δ*pipD S.* Agona showed the least effect to the kidneys compared to other bacterial treatments, while unattenuated *S.* Typhimurium was showed to exert the most effect to the histopathology of kidneys.

A significant increase in plasma neutrophil level was observable for tumour-bearing mice receiving all four bacterial treatments. *S.* Typhimurium showed the highest increase of plasma neutrophil levels, followed by *S.* Agona. Tumour-bearing mice treated with the Δ*sopB*Δ*sopD*Δ*pipD* strains showed a lower increase of plasma neutrophil levels when compared to its unattenuated bacteria counterparts. Neutrophils are the most abundant polymorphonuclear leukocyte found in blood and are important as the first line of defence against inflammation and infections (40,41). In response to physiological stresses such as infection, the neutrophils are released by the bone marrow in a regulated manner to ensure stable homeostatic levels in the blood which are important for a stable neutrophils blood count (42,43).

Neutrophils are also observable in the meninges of tumour-bearing mice; however, there are no signs of inflammation or necrosis observable (Figure 6F-J). A study demonstrated that mice inoculated orally with *S.* Typhimurium showed signs of brain infection and meningitis (44). In another study, which utilises in vitro model, it was observed that *S.* Typhimurium was able to adhere and successfully invade the microvascular endothelial cells of the brain which further supports the idea that blood-borne pathogens are able to interact with the cerebral endothelial cells and cross the blood-brain barrier (45). The inflammatory response is provoked once the bacteria starts to replicate within the central nervous system.

By looking into the effects of the Δ*sopB*Δ*sopD*Δ*pipD* strains (plasma testosterone, AST and ALT, plasma neutrophil and histopathology analysis), it is observable that these attenuated strains exert less systemic effects to the tumour-bearing mice. It had been shown that double mutations of Δ*sopD*Δ*pipD* had shown minimal attenuation effects on the systemic infection in mice even when it showed great attenuation of secretory responses in a bovine ligated ileal look model of enteritis (46). A study reported by Jones et al. has shown that double mutations of Δ*sopB* and Δ*sopD* displayed an additive attenuation effect, as it induced even lower secretory and inflammatory responses in infected ileum than the single mutants (47). In another study by Gwee et al., it was reported that the attenuation of Δ*sopB* and Δ*sopD* in *S.* Agona showed no improvement of mice survivability when compared to tumour-bearing mice treated with the unattenuated parental strains (32). From the findings of this study, the addition of the combination of Δ*sopB*Δ*sopD*Δ*pipD* attenuation seems to overcome this and showed improved attenuation effects on the systemic infection in mice.

CONCLUSION

The findings from this study suggest that Δ*sopB*Δ*sopD*Δ*pipD S.* Agona strain shows great potential to be utilised as a tumour inhibition agent since it has shown to exert less systemic effects to the tumour-bearing mice treated with it while showing promising improvement in its tumour inhibition capabilities.

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