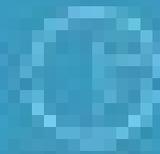


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The Effect of Acute and Chronic Infection-Induced by AvrA Protein of *Salmonella typhimurium* on Radical Oxygen Species, Phosphatase and Tensin Homolog, and Cellular Homolog Expression During the Development of Colon Cancer

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Abstract

AIM: The study aimed to analyze the effect of AvrA effector protein of *Salmonella typhimurium* in inducing colon cancer through increased of radical oxygen species (ROS), phosphatase and tensin homolog (PTEN), and avian myelocytomatosis virus oncogene cellular homolog (c-Myc) expression, in mice model of colorectal cancer.

METHODS: This study used Balb/c mice which were divided into four types of groups: Negative control, exposed to azoxymethane (AOM), treatment with AOM, and AvrA (AOM+AvrA), and treatment with AOM and *S. typhimurium* (AOM + *S. typhimurium*). Each type consists of a 1-week treatment group and a 12-weeks treatment group, with a final number of eight groups. *S. typhimurium*-specific protein (AvrA) was isolated and then injected to AOM + AvrA groups (40 µg/50 µl), intraperitoneally. *S. typhimurium* was administered orally to AOM + *S. typhimurium* groups. ROS production in peripheral blood mononuclear cells was measured by flow cytometry. PTEN and c-Myc expression in colon tissue were detected through immunohistochemistry.

RESULTS: The study showed that ROS production was higher in the 12-week AOM + *S. typhimurium* treatment group compared with other 12-week treatment groups ($p < 0.05$). AOM + AvrA and AOM + *S. typhimurium* groups demonstrated a decrease of PTEN expression and an increase of c-Myc expression in colon tissue, compared to AOM groups, both in 1-week and 12-weeks treatment ($p < 0.05$).

CONCLUSION: AvrA effector protein from *S. typhimurium* increased ROS production and c-Myc expression while suppressed PTEN expression as markers of colorectal cancer, both in acute and chronic infections.

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Introduction

Colorectal cancer is a major contributor to morbidity and death in the world. The incidence of colorectal cancer is the third-highest in the world and the second most deadly after lung cancer. In 2018, there were an estimated 1,006,019 men and 794,958 women living with colorectal cancer in the world [1]. Colorectal cancer is triggered by several factors including carcinogenic chemicals, ultraviolet light, viruses, and bacteria facilitating gene mutations [2].

An increased risk of colorectal cancer was found among patients diagnosed with severe salmonellosis [3]. *Salmonella* has several T3SS organelles such as SopE, SptP, SopB, SipA, and SipC, which contribute to its pathogenicity. AvrA is another *Salmonella* effector protein that has not been widely known. The function of AvrA is presumed to be the same as other effector proteins [4]. The mechanism of AvrA effector in the pathogenesis of *Salmonellosis* remains not clearly understood. Early-stage of *Salmonella* infection is characterized by activation of macrophages, inflammation of infected tissue, and production of

gamma interferon (IFN) by several cells. T CD4⁺ cells play an important role in controlling *Salmonella* infections, together with CD8⁺ and T γ δ cells [5].

The increased immune system activity stimulates cytokine synthesis [6], which, in turn, triggers both chronic inflammation and radical oxygen species (ROS) production. Uncontrollable inflammation and ROS in host cells ultimately damage the tissue occupied by *Salmonella* during the infection [7]. *Salmonella* or its effector proteins induce inflammation and ROS by activating the JAK-STAT, JNK, and Wnt pathways in stem cells. Activated pathways cause uncontrolled cell cycles which lead to increased risk of malignancy. Cancer stem cells will stick (homing) on enterocytes because of chemoattractants produced by the inflammation process. Effector proteins of *Salmonella* activate Toll-Like receptor-4 therefore it blocks phosphatase and tensin homolog (PTEN) and stimulates MyD88. The activation of both proteins, followed by phosphorylation of NF- κ B, leads to nuclear translocation of NF κ B subunits to stimulate protein synthesis. Some of the synthesized proteins are proteins involving in the proliferation (cellular homolog [c-Myc], and cyclinD1), apoptosis (FasL, BAX, and Caspase), and angiogenesis (VEGF and c-IAP2) [8]. The study explored the effect of AvrA's effector on ROS production in peripheral blood mononuclear cell (PBMC) as inflammation indicator, and PTEN and c-Myc expression in colon tissue as colon cancer markers. AvrA protein used for this study was isolated from *Salmonella typhimurium* as the best-studied serovar of Salmonellosis.

Materials and Methods

Ethics statement

This study was approved by the Research Ethics Commission of the Faculty of Medicine, Universitas Brawijaya, Indonesia, as stated in the Code of Ethics for Research, number 154/EC/KEPK-53/05/2019. All efforts were made to minimize suffering.

S. typhimurium culture

S. typhimurium was obtained from American Type Cell Culture with Catalog Number 2354 Lot Number # 58105535. Bacteria isolates were propagated on MacConkey medium and then incubated at 37°C temperature for 18–24 h. The culture from the MacConkey medium was transferred to a biphasic medium consisting of BHI liquid medium and TCG agar slant medium. The culture was incubated at 37°C temperature for 24 h.

Isolation of *S. typhimurium* bacterial protein

After *S. typhimurium* had been cultured, centrifugation was carried out to separate bacteria

from the media. The media were removed and the cell resuspended with phosphate buffer saline (PBS). This step was repeated 3 times. The last precipitate was resuspended with TRIzol reagent to isolate bacterial proteins. The AvrA protein was identified as the presence of 34 kDa protein, detected by mouse anti-AvrA polyclonal antibody (Abcam, USA), through Western blotting.

The bands corresponding to the proteins were excised from the gels and transferred to a cellophane membrane. The gel solution was electro-eluted with Horizontal Electrophoresis Apparatus for 25 min. The results were dialyzed with a sterile PBS for 2 × 24 h. The concentration of the isolated protein was calculated with nanodrop.

Animal groups

The experiment was performed using male Balb/c mice (Pusvetma, Indonesia) that were 3 weeks old and had \pm 50 g body weight. The animal randomly assigned to four treatment groups: The negative control group which was not exposed, the positive control group which was only exposed to azoxymethane (AOM), the group exposed to AOM + AvrA, and the group exposed to AOM and *S. typhimurium* (AOM + *S. typhimurium*). Each group was divided into subgroups: A 1-week and a 12 weeks duration of treatment. The 1-week duration treatment depicted acute inflammatory mice model while the 12-week duration treatment depicted chronic inflammatory mice model [4].

Salmonella or AvrA-infected colorectal cancer mouse model

A series of treatments were given regularly every week. Thus, the number of treatment series was proportional to the duration of the treatment, whether 1 week or 12 weeks. Mice have fasted for 4 h. Afterward, the mice were given 7.5 mg/mouse streptomycin (100 μ l sterile solution) and continued with water and food supply *ad libitum*. Exposures were conducted 20-h after streptomycin treatment, with preceded 4 h fasting. AvrA protein exposure was given to AOM + AvrA group, intraperitoneally (40 μ g/50 ml). *S. typhimurium* was given orally to AOM + *S. typhi* group, as 100 μ l suspension of 1×10^6 CFU in Hank's balanced salt solution. Both the negative control group and the AOM group were treated with sterile Hank's balanced salt solution, orally. AOM (TCI, Tokyo) was given through oral gavage to all groups (10 mg/ml), except for the negative control group.

Measurement of colon cancer antigen (CCA)

Before the main experiment, we explored the effects of AOM, *S. typhimurium*, and AvrA on

colon carcinogenesis, using four groups of mice given the same type of exposures with the main experiment. Blood samples were collected on the 14th day of treatments. Mouse CCA was detected using Sandwich- enzyme-linked immunosorbent assay (ELISA) kit (Elisa Genie, UK). The serum was added to CCAs-antibody coated wells. Then biotinylated detection antibody specific for Mouse CCA and Avidin-Horseradish Peroxidase conjugate was added to each well successively and incubated. The substrate solution was added to each well. The enzyme-substrate reaction was terminated by adding Stop Solution and the color turns yellow. The optical density was measured spectrophotometrically at 450 nm.

Measurement of PBMC intracellular ROS

Intracellular ROS production was measured in PBMC. The cells were washed twice with PBS before being shaken slowly. The final precipitate was added with 100 μ L PBS and 1 μ M carboxy-H₂DCFDA (TRC, Canada). The cells were incubated in dark conditions for 60 min at room temperature. ROS testing was analyzed with flow cytometry (FACS Calibur, BD) on the FL1 channel [9].

PTEN-1 and c-Myc expression in colon tissue

The expression of PTEN and c-Myc was observed in the colon tissue slides by immunohistochemical staining. Each protein expression was detected using mouse anti-PTEN monoclonal antibody or mouse anti-c-Myc monoclonal antibody (IGEIA, Indonesia) in fetal bovine serum (1:100). Their expression appeared as brown precipitates in the colonic crypt regions [10], [11]. The expression was analyzed using ImmunoRatio® software. The results were presented as the percentage of positively-stained areas out of the total nuclear area.

Data analysis

The statistical analysis was performed with SPSS 23. Kruskal–Wallis (non-normal distribution) or one-way ANOVA (normal distribution) tests were applied to compare variables within the same duration. A comparison of each treatment group between different duration was using Mann–Whitney test (non-normal distribution) or independent t-test (normal distribution). Further comparisons between all groups were utilizing Kruskal–Wallis or one-way ANOVA continued with *post hoc* multiple comparisons LSD. $p < 0.05$ was accepted as statistically significant.

Results

CCA level in plasma

CCA concentration in plasma was detected higher in AOM exposure than in negative controls. Exposure to AvrA and *S. typhimurium* increases CCA higher than the AOM group. The highest plasma CCA levels detected in AOM + *S. typhimurium* group, despite not significantly different than the AOM + AvrA group (Figure 1).

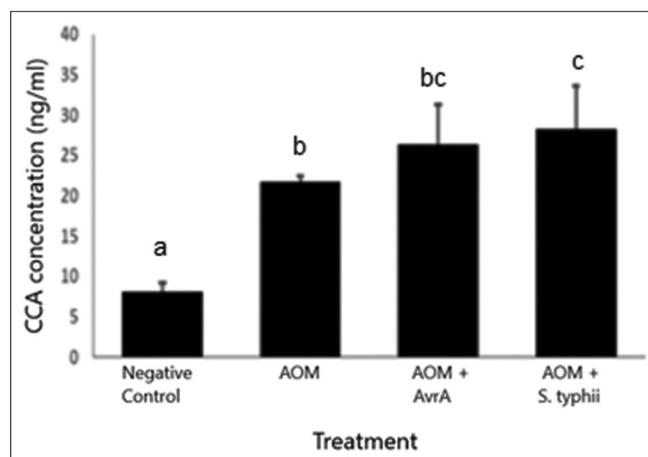


Figure 1: The results of measurements of plasma colon cancer antigen (CCA) levels after 2-weeks treatments. CCA level exploration was done before the main experiment a, b, or c labels indicate that there was no significant difference between groups with the same label, as tested by multiple comparisons LSD

ROS production in the PBMC of colorectal cancer model mouse

Compared with the negative control group, AOM treatment in the AOM group induced a significant increase in ROS production after a week, but there was no difference at 12 weeks of treatment duration (Figure 2). Neither the AOM + AvrA nor AOM + *S. typhimurium* group experienced a significant change in ROS production compared to the AOM group at 1-week treatment. After 12 weeks of treatment, ROS production was induced higher in the AOM + AvrA group compared to the AOM group, and the highest in the AOM + *S. typhimurium* group (Figure 3). ROS production in the AOM + *S. typhimurium* group almost doubled at 12 weeks compared to 1-week treatment (Table 1).

Table 1: Percentage of ROS production in PBMC of 1-week and 12 weeks treatment groups

Treatment	Weeks		p
	1	12	
Negative control	13.22 ± 8.52	4.86 ± 2.30	0.251***
AOM	23.32 ± 1.87	3.51 ± 0.61	0.000**
AOM+AvrA	21.19 ± 8.30	24.76 ± 4.71	0.754***
AOM+S. typhimurium	23.12 ± 2.86	45.78 ± 2.93	0.000**
p	0.052*	0.001*	0.000*

Data were represented as Mean ± SEM. *Kruskal–Wallis test, **Independent t-test, ***Mann–Whitney test. *Salmonella typhimurium*: *S. typhimurium*, ROS: Radical oxygen species, AOM: Azoxymethane, PBMC: Peripheral blood mononuclear cell.

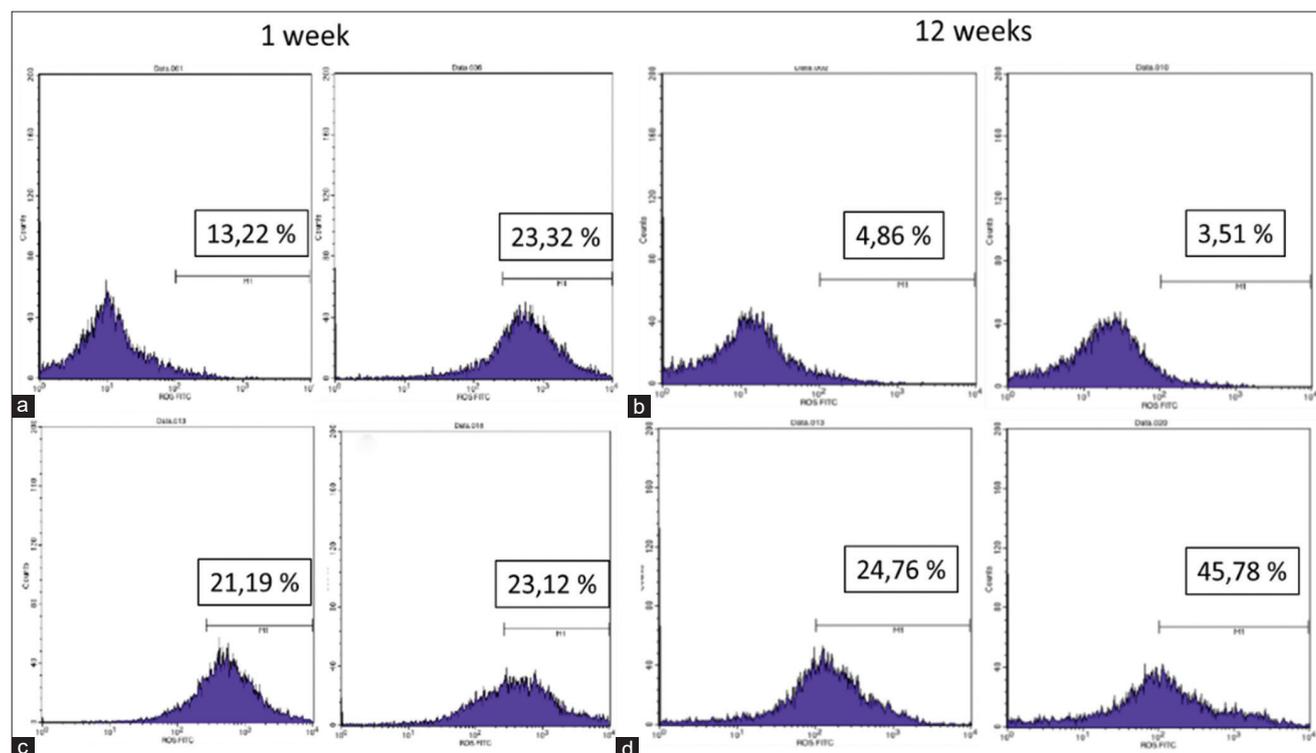


Figure 2: The results of radical oxygen species flow cytometry among the study groups. (a) Negative control; (b) azoxymethane (AOM); (c) AOM + AvrA and (d) AOM + *Salmonella typhimurium*

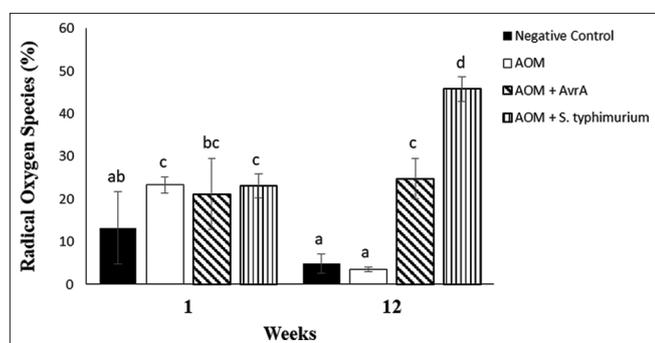


Figure 3: Comparison of radical oxygen species production between study groups a, b, c, or d labels indicates that there was no significant difference between groups with the same label, as tested by the Mann-Whitney test

PTEN expression in the colon tissue of colorectal cancer model mouse

AOM significantly increases PTEN expression in both 1-week and 12 weeks of treatment. The administration of AvrA or *S. typhimurium* attenuated PTEN expression compared to the AOM group in both treatment durations (Figure 4). The reduction of PTEN expression in the AOM + *S. typhimurium* group was lower than in the AOM + AvrA group. Compared to 1-week treatment, a longer duration of 12 weeks elevated PTEN expression on AOM + AvrA and AOM + *S. typhi* groups (Table 2). Nevertheless, the expression of PTEN in these two groups was still lower than the AOM group for the same treatment duration (Figure 5).

Table 2: PTEN expression of the colon tissue of colorectal cancer model mouse treated with AvrA

Treatment	Weeks		p
	1	12	
Negative Control	22.98 ± 8.57	26.75 ± 7.73	0.487**
AOM	85.52 ± 5.43	74.62 ± 8.78	0.046**
AOM+AvrA	52.42 ± 7.59	62.48 ± 4.03	0.031**
AOM+S. typhimurium	38.88 ± 2.54	49.53 ± 3.98	0.001**
p	0.000*	0.000*	0.000*

Data were represented as Mean ± SEM. *One-way ANOVA test. **Independent t-test. *Salmonella typhimurium*: *S. typhimurium*, PTEN: Phosphatase and tensin homolog, AOM: Azoxymethane.

c-Myc expression in the colon tissue of colorectal cancer model mouse

AOM exposure increased c-Myc expression in AOM groups compared to negative controls at 1-week and 12 weeks of treatment (Table 3). c-Myc expression in the AOM + AvrA and AOM + *S. typhimurium* groups had increased compared to the AOM group in both treatment durations. After 1-week treatment, AvrA administration increased c-Myc expression higher than the *S. typhimurium* administration. But at 12 weeks' duration, the rise in c-Myc expression in both groups

Table 3: The expression of c-Myc of the colon of male mice treated AvrA *S. typhimurium* for 1 and 12 weeks

Treatment	Weeks		p
	1	12	
Negative control	35.12 ± 3.31	19.05 ± 4.41	0.000**
AOM	51.21 ± 11.41	50.59 ± 6.37	0.918**
AOM + AvrA	83.25 ± 4.08	75.67 ± 13.53	0.265**
AOM + <i>S. typhimurium</i>	70.35 ± 9.61	75.96 ± 6.32	0.307**
P	0.000*	0.000*	0.000*

Data were represented as Mean ± SEM, *p < 0.05 versus control. *One-way ANOVA test. **Independent t-test. *Salmonella typhimurium*: *S. typhimurium*, c-Myc: Cellular homolog, AOM: Azoxymethane.

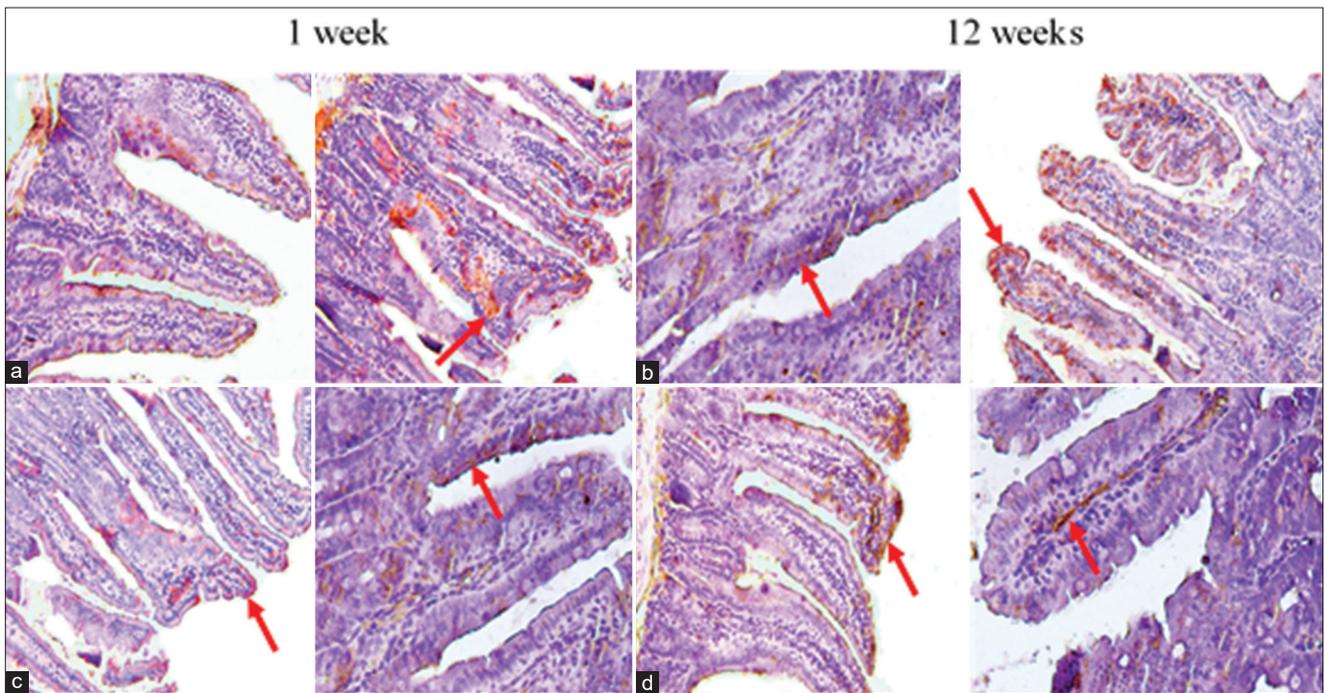


Figure 4: The effect of AvrA exposure on phosphatase and tensin homolog (PTEN) expression of the colorectal cancer model mouse. PTEN expression in colon tissue was detected by immunohistochemical staining. (a) control; (b) azoxymethane (AOM); (c) AOM + AvrA; (d) AOM + *Salmonella typhimurium* groups (Nikon Eclipse 100 photomicroscope with 400 \times)

was not significantly different. In the three treatment groups, the comparison of c-Myc expression between 1-week and 12 weeks duration showed no significant difference (Figure 7).

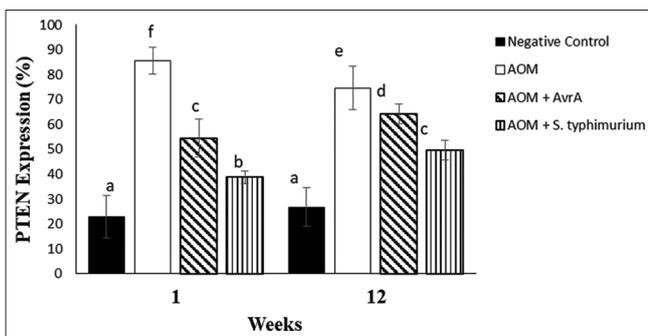


Figure 5: Comparison of phosphatase and tensin homolog expressions in the colon tissue between study groups a, b, c, d, e, or f labels indicate that there was no significant difference between groups with the same label, as tested by multiple comparisons LSD

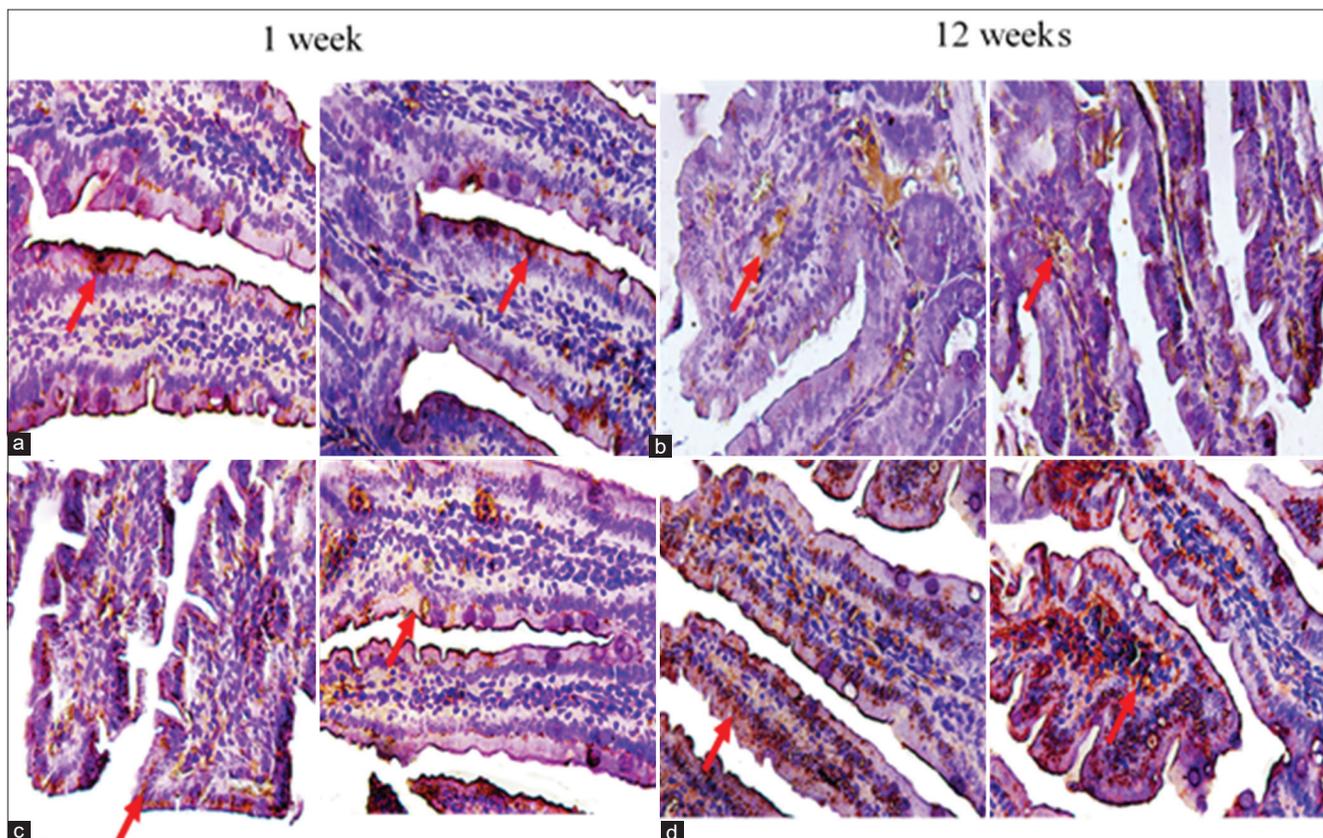
Discussion

Recently, growing evidence shows a correlation between *Salmonella* infection with the development of colon cancer [12], [13]. Inflammation is agreed on as a linking mechanism between *Salmonella* infection and colon cancer. However, further research identified *Salmonella* effector proteins which directly influence biological processes of the host [14], [15]. This study explored the effect of the *Salmonella* effector protein, AvrA, in inducing colon cancer in chronic and acute inflammation, using AOM -induced colorectal cancer models.

AOM is commonly used to induce colon cancer model [16]. In our study, exposure to AOM for 1 week increased ROS production in peripheral blood monocyte cells (PBMC). AOM undergoes metabolic activation into DNA-reactive products, which can alkylate macromolecules in the colon, and eventually induce DNA damage and micronucleus formation [17]. This event activates a cyclic GMP-AMP synthase – interferon gene stimulator response, which triggers transcription of inflammatory genes [18]. Immune response stimulates the production of chemokines and ROS. The immune response in the form of ROS production can be found in various peripheral mononuclear leukocytes, for example, activated monocytes [19], NK cells [20], T lymphocytes [21], and B lymphocytes [22].

The result showed that chronic exposure to AOM orally did not increase ROS production in PBMC compared with negative control, which might be attributed to adaptation of intracellular antioxidants and metabolic reprogramming [23]. Interestingly, the addition of AvrA to AOM exposure kept ROS production high in both acute and chronic treatment. Meanwhile, the combination of *S. typhimurium* and AOM increases ROS production almost double the acute treatment. These results indicate that *S. typhimurium* could stimulate acute and chronic inflammation and AvrA was one of *S. typhimurium* components that trigger inflammation.

The role of AvrA in the process of infection and inflammation might be associated with the development of colon cancer in the mouse model. In this study, AvrA exposure provided an additional inflammatory burden, which was demonstrated by higher ROS production,



AQ3 **Figure 6:** Immunohistochemical results of cellular homolog expression in each group in mice model of colorectal cancer induced with azoxymethane. The decreased phosphatase and tensin homolog expression in (a) control; (b) azoxymethane (AOM); (c) AOM + AvrA; (d) AOM + *Salmonella typhimurium* groups (Nikon Eclipse 100 photomicroscope with 400×)

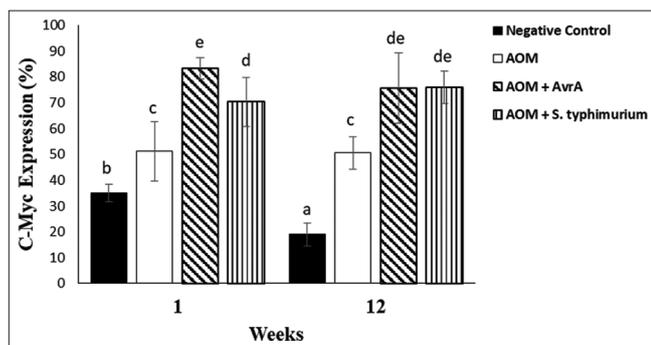


Figure 7: Comparison of cellular homolog expressions in the colon tissue between study groups a, b, c, d, or e labels indicate that there was no significant difference between groups with the same label, as tested by multiple comparisons LSD

especially in chronic exposure. ROS produced by inflammatory cells is converted to secondary products O_2^- and NO_2^- , as oxidizing and nitrating agents that easily damage DNA and thus accelerate mutagenesis [24]. AvrA also activates STAT3 pathways which are promoting inflammation-associated colonic tumorigenesis [25]. Inflammation activates many pathways including the NF- κ B pathway that synergize with Wnt signal activation which maintains stemness and activates cancer stem cells [26], [27].

The results suggested that AOM alone as well as in combination with AvrA or *S. typhimurium*, facilitated colorectal carcinogenesis, as observed in

elevation of plasma CCA concentration after 2 weeks of the treatments. Compared to AOM exposure, AvrA exposure induced a higher concentration of CCA which supported the hypothesis that AvrA triggers colorectal carcinogenesis. However, AvrA's contribution to this process might act as the main effector protein of *S. typhimurium* which facilitates colorectal carcinogenesis. This notion was evidenced by the concentration of CCA in the AvrA and *S. typhimurium* treatments that were not significantly different.

PTEN (deleted on chromosome 10) is a negative regulator of cell growth and survival signaling pathways [28]. In this study, acute and chronic administration of AOM induced PTEN expression. AOM given by oral gavage might cause DNA damage, which, in turn, triggers increased PTEN expression as part of DNA-damage repair mechanism. In the nucleus, PTEN promotes the stability and transcriptional activity of the tumor suppressor p53 by directly associating with p53. PTEN is also found to collaborate with E2F to induce the expression of Rad51 and thus enhance DNA repair [29], [30]. PTEN also induces expression of multiple pro-apoptotic members of the Bcl2 family, stimulating expression of death receptor ligands, or by enhancing levels of various cyclin-dependent kinase inhibitors [28].

This study demonstrated that *S. typhimurium* and AvrA administration suppressed PTEN expression.

The mechanism of decreased PTEN expression in colorectal carcinogenesis can occur epigenetically, genetically, post-translational modification, or mislocalization. Genetic mutations and a decrease in the number of PTEN gene copies are less common [31], [32].

S. typhimurium exposure caused significant suppression of PTEN compared to AvrA, which showed that AvrA had a partial role in suppressing PTEN. This result may be explained by *S. typhimurium* as a whole organism that triggers more severe inflammation [33], and the presence of other proteins, such as typhoid toxin-cytolethal distending toxin, which also affects DNA damage and carcinogenesis [34].

The results of this study suggest possible mechanism of AvrA in apoptosis and cell cycle arrest, which is in addition to its acetyltransferase activity which deactivates p53 [35], AvrA might also inhibit those events through PTEN suppression. Depression of PTEN expression will increase the activation of the phosphoinositide 3-kinase/protein phosphatase 2A pathway which then activates β -catenin [36]. Besides, AvrA activates Wnt/ β -catenin pathway in intestinal stem cells through β catenin phosphorylation (increasing activation) and deubiquitination (decreasing degradation) [37], thereby supporting the effect of Salmonella on colorectal carcinogenesis.

c-Myc gene is a proto-oncogene which produces transcription factor. *c-Myc* protein can activate or suppress various target genes involved in cellular function, including cell cycle, survival, protein synthesis, and cell adhesion [38]. Overexpression of *c-Myc* was observed in 70–80% of colorectal cancers and was associated with low survival of CRC patients [39]. Increased *c-Myc* expression facilitates cancer characteristics development including uncontrolled proliferation, resistance to cell death, genomic instability, immune escape, angiogenesis, and metastasis [40].

The previous studies showed that exposure to AvrA-expressed Salmonella did not increase total *c-Myc* [41]. In contrast, our study demonstrated that exposure to isolates of AvrA protein increased *c-Myc* expression in both acute and chronic treatment. In acute exposure, AvrA stimulated higher *c-Myc* expression than *S. typhimurium*. Meanwhile, in chronic exposure, increased *c-Myc* expression due to AvrA and *S. typhimurium* was comparable. This result indicates that AvrA plays a major role in increasing *c-Myc* expression in ST-induced colorectal carcinogenesis. The elevation of *c-Myc* might be related to AvrA's ability to intensify β -catenin activation [37], [42]

Elevation of *c-Myc* expression in solid cancer occurs through various mechanisms such as gene amplification and chromosomal mutation [39], which may be related to oxidative stress and damage [43], [44]. Inflammation is another mechanism that can activate

the *c-Myc* expression, through some inflammatory cytokine (Interleukin [IL]-6 and tumor necrosis factor- α) stimulation [45]. In turn, *c-Myc* may stimulate the pro-inflammatory signaling pathway and cytokines, including IL-6, IL-8, IL-1 β , CCL2, and CCL20. These events provide a suitable niche for the transformation of stem cell phenotype into tumor progenitor [46]. *c-Myc* also contributes to maintaining self-renewal and chemoresistance properties of colon cancer stem cells [47].

Conclusion

AvrA protein effector played an important role in the inflammation – carcinogenesis sequence of colorectal. In acute and chronic Salmonella infection, AvrA had a partial role in suppressing PTEN expression and act as the main effector in regulating *c-Myc* overexpression, leading to colorectal carcinogenesis. Therefore, AvrA may be a new target for the prevention and treatment of Salmonella-associated colorectal cancer.

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