International Journal of Advanced Research and Publications
ISSN: 2456-9992

Cyto-Pathological Profile Of Intestinal Epithelial Cells Of Chicks At 3 Hours Age Post Invasion By Salmonella Enteritidis

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Abstract: Salmonella often interferes with host cell functions such as actin polymerization and signal transduction to induce apoptosis and death. Salmonella enteric serotype enteritidis when targeted host cell it secretes effector protein, then proliferate into the cells concurrently leading to apoptosis, the effector protein will interact with that of the host cells. In the current study experimental newly hatched chick was taken as a model, twelve chicks were inoculated with salmonella cells at dose 10^{-5} intercrop. Three hours post-inoculation the chicks were sacrificed. Histopathological sectioning of intestine from affected chicks revealed, degenerative changes, infiltrate of the heterophil and mononuclear cell, as well as sever sloughing epithelial cells into the intestinal lumen. Cytopathology using the flow cytometer analysis showed that epithelium cells were invaded by Salmonella enteritidis for 3 hours. The flow cytometer succeeded to detect singlet epithelial cells that invaded by salmonella pathogen. Also, it described the effects of viable salmonella cells that rendering epithelial lining under pressure of invasion and induction of apoptosis as well as the cellular immunity was stimulated at 3hours of age. The study conducted on the processes of phagocytosis, kill and/or degradation against salmonella was not neutralized when compared with the destruction of intestinal epithelial cells and sluffing into lumen that means the losses in chicks started at 3hours of age.

Keyword: Epithelial Cells, Cytopathology, Newly Hatched Chicks, Salmonella enteritidis Corresponding author: amjadzubair79@gmail.com. Co-author: wawma81@hotmail.com.

1. Introduction

Avian salmonellosis is a septicemic disease affecting primarily chickens, turkeys, and other birds such as quail, pheasant, ducks, peacock, and Guinea fowl. Wild birds are also susceptible, (Shivaprasad, 2000). Two serotypes, Salmonella pullorum,
and Salmonella gallinerum are adapted to infect poultry flocks. Chicken is the most important reservoir of salmonella that can be transmitted through food chain to humans. Carrier bird serve as route of transmission of salmonella vertically and horizontally. The infection in adult birds is asymptomatic, occurred during the first few weeks of life. Its clinical picture is similar to that of pullorum disease (loss of appetite, nervous symptoms, and blockage of the cloaca with diarrheal fecal matter). Nodules may be present in the muscle of the ventriculus, gizzard, and rectum and occasionally in the wall of the ceca that may contain caseous cores. Cell injury states any biochemical or cell structure alteration that impairs the ability of a cell to function normally. Such an injury may be mild, transient, and reversible or it may be of such severity that becomes irreversible with time. Cell death occurs in two biochemically and morphologically distinct ways, Accidental cell death and apoptosis. Finally, necrosis refers to the enzymatic degradation of the nucleus and cytoplasm that define cell death. Typically, these changes cannot be seen for 12 to 18 hours after a cell death. Cell death and accidental cell death (simply as necrosis) are the most often recognized type and although it has numerous causes, they typified by biochemical and morphological alterations resulting from anoxia. The other type, often referred as ‘‘programmed cell death’’ termed apoptosis results from activation and transcription of a specific gene. The types of injury consider a series of events unfold that ultimately lead to loss of cellular membrane integrity and acute cellular swelling. The swelling of cells is one of the earliest recognizable changes following cellular injury (Jones, et al., 1997).

2. Material and method

Experiments model

Salmonella strain used in this study was isolated from chicken in Central Veterinary Research Laboratories-origin Rabak, Sudan. The strain was assessed and confirmed to induce diarrhea as well as shedding in the facaes. Then strain was labelled by Yellow Fluorescent Protein (YFP) had (ex/em. 525/538). The permeability of YFP into the cytosol of salmonella was simultaneously labelled to plasmid by transformation method described by Chang, et al., (2017). Newly hatched, Cob breed chicks were obtained from Mico Hatchery (Dajin for poultry production Co. LTD), they were salmonella-free, healthy and the general status was suitable as a model. The method of rearing and feeding used as described by Barrow, (1991). The inoculum dose was kept on a 10 ml fresh culture of peptone broth (Oxoid) and incubated at 42C^{o}. The viable count of the infective dose was adjusted to 10^{-5} after serial dilution as described by Riberio, et

al., (2005). The inoculum dose was adjusted to 0.5 ml performed by syringe connected to cannula contain a fixable tube (IV cannula). The inoculum dose was gently given intercrop, and the clinical signs was observed and recorded. Scarification of eleven inoculated chick was performed after 3 hours. Then post-mortem examination and the identification of necropsied chicks were recorded

.**Bacterial Investigation**
Samples for bacterial examinations were collected from lumen of the small intestine to detect intracellular salmonella.

Histopathological Study
Five centimetres from the characteristic lesion of enteritis were collected into 10% formaldehyde as fixative. Stages of trimmed, processing, and sectioning and staining were prepared at Pathology Department, Central Veterinary Research Laboratories, Soba, Sudan.

Flow Cytometer Study
Another Five centimetres from the same characteristic lesion were prepared by Annexin-V FITC kits (MACS, Miltenyi Biotec) as described by manufacturer to detect viable cells, apoptosis, and necroptosis of intracellular salmonella.), Apoptotic cells were stained positively for Annexin V-FITC that binds to phosphotidylserine (PS), while negative for staining with Propidium iodide (PI). Dead cells were stained positive for Annexin V-FITC and PI, whereas viable cells were negative for both Annexin V-FITC and PI. (Miltenyi Biotec) Epithelial cells required to labelling by fluorescence and suitable volume were up to 10^6 total cells. When working with fewer than 10^6 cells, use the same volumes as indicated. When working with higher cell numbers, scale up all reagent volumes and total volumes. Cells number was determined. 10^6 in 1ml of 1× Binding Buffer (250µl 20× Binding Buffer Stock Solution with 4.75ml of sterile DW) was washed. Washing was repeated. Cell pellet in 100µl of 1×binding Buffer was resuspended per 10^6 cells. 10µl of Annexin V-FITC was added per 10^6 cells. Mixed well and incubated for 15 minutes in the dark at room temperature. Cells were washed by adding 1mL of 1X binding Buffer per 10^6 cells and centrifuge at 300 X g for 10 minutes. Supernatant was aspirated completely. Then washing was repeated. Cell pellet in 500µl of 1X Binding Buffer was resuspend per 10^6 cells. Finally, 5µl of PI solution immediately added prior to analysis by flow cytometry.

Statistical analysis
The significance of eleven samples was determined by applying to the SPSS computer software program. The statistical analysis aimed to differentiate and detect the effectiveness of time by multiple comparisons and ANOVA tests as well as X-median to determine the dimension of the epithelium was affected.

Results

Clinical symptoms
Clinical signs of the inoculated chick after 3 hours were found to be activeness, good appetited, and no sudden death in comparison to the negative control. Neither diarrhea nor pasty vent were observed.

Gross Pathology
Enteritis and congestion were observed in all birds with variation in the severity. Very slight Pericarditis was notice after 3 hours.

Histopathological finding:
The selected samples from enteritis part were generally revealed degeneration and desquamation of lining epithelium cells. Infiltration of heterophils and other mononuclear cells in mucosa and submucosa which lead to atrophy of intestinal glands with an invasion of fibroblast. Severe Sluaffing of epithelial cells were shown within lumen during 3 hours, figure (1).

Detection of Salmonella enteritidis invasion by flow cytometer.

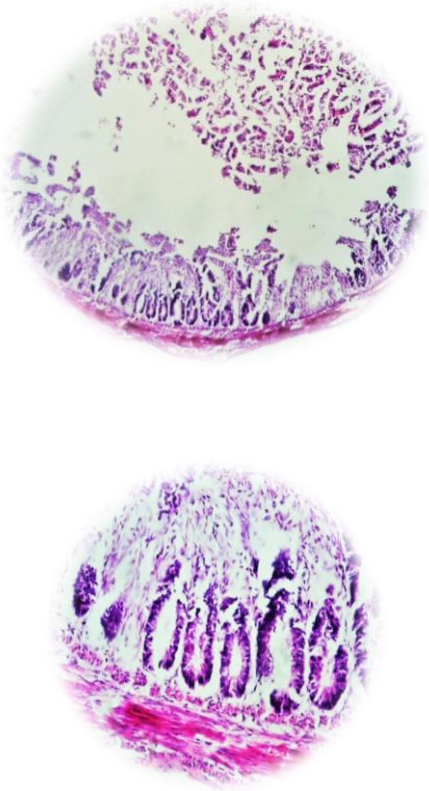


Figure (1) intestinal Histopathology for Group 3 hours; **A** cross section of intestinal tissue contain degenerative changes, desquamation of lining epithelial cells; sluffing of epithelial cells into lumen, descripted tight junction between cells, atrophy of intestinal glands, inserted fibroblast and disappear macrophages.

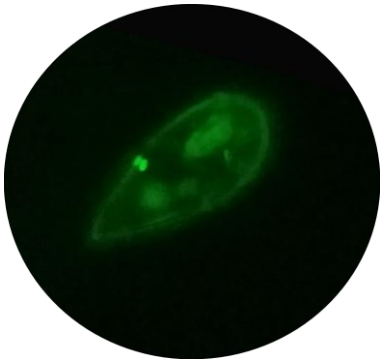
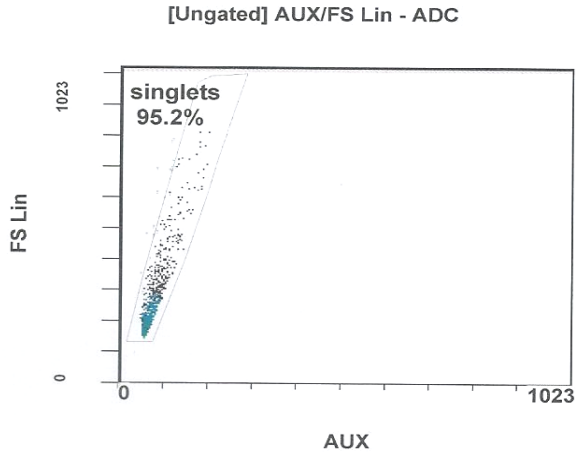
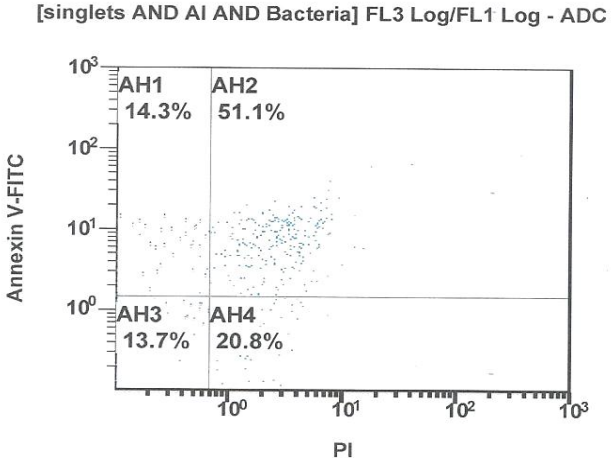


***Figure 2**, fluorescence microscope, A-epithelium Cells, B- Salmonella cell invaded*

Detection of invasion of pathogen was applied by fluorescence microscope that ensure salmonella-yellow fluorescent protein found inside epithelium cell, figure (2). Flow cytometer confirmed the epithelial cells that invaded by salmonella-yfp. Figure (3). Assessment of Epithelial cells status The flow cytometer detected and differentiated singlet cells containing salmonella-yfp figure 3 (A). The epithelial were differentiated according to cell cycle (viable cells, apoptotic cells, dead apoptotic cells, and irreversible dead epithelial cells) with emphasis intracellular salmonella-yfp. The differentiation of apoptosis stage from dead cell was applied by indicators Annexin V FITC and Pi, any cells out of detection by indicator was considered viable cells, figure 3 (B)





The ANOVA test; describe the effect of restricted time (3hours) on invasion of salmonella into epithelial cells table (1).
The significance between variables (Live Epithelial Cells, Live Apoptotic, Dead Apoptotic and Dead -Irreversible) in 3 hours post-inoculation was revealed strong relationship between live epithelial cells, live apoptotic and dead apoptotic epithelial cells as well as dead epithelial cells-irreversible, while not significance between live apoptotic epithelial cells and dead apoptotic epithelial cells was observed, table (2).

Discussion

Salmonella causes a wide range of diseases in human and animal hosts ranging from self-limiting enteritis to systemic infections (Coburn, et al., 2007). Salmonella enterica serovars enteritidis are broad host-range pathogens, infect both human and animal hosts (Mastroeni and Maskell, 2006). Chicken ranked the largest reservoir host for Salmonella enteritidis, (Kimura, et al., 2004; Patrick et al., 2004). Salmonella enteritidis is transmitted via contamination of faces. Young chickens at 2 weeks of age often develop gastroenteritis and systemic disease with varying degree of mortality. Most adult hens become colonized with Salmonella enteritidis but typically remain asymptomatic carriers with intermittent faecal shedding. Generally, Salmonella enterica replicates inside the epithelial cell and consequently interfer with host cells functions in form of actin polymerization, signal transduction, and apoptosis. Then salmonella migrates outside epithelial cell, and induces death. (Hansen-wester and Hensel, 2001; Ghosh, 2004). The fact that newly hatched chicks of 3hours ages was not fully developed an intestinal tract and not adapted to feed consumption may explain absence of diarrhea and the pasty vent during early stage of experiment. Although apoptosis occurred in this stage, no significance relationship between live apoptotic and dead apoptotic cells. The current study found the epithelial cells was in challenged directly by competition, salmonella to be survive before proliferation and the clear disturbances that occurred in cytosolic essential electrolytes. As no data were found concerning standard measurement of intracellular traces element in poultry, it worth mentioning that the study it was the prior one in comparing electrolyte disturbances with four negative control (range of minimum and maximum concentration, data not show). Accidental swelling of dead cell appeared in epithelial cells that invaded intracellularly by salmonella. This considered as negative impact of salmonella on maintenance of epithelium, accelerating induction of apoptosis and/or necroptosis process. As descriptive form, qualitative evidence was possible to determine the correlation between apoptosis and necroptosis through applying flow cytometer technique. The scholar was successful in differentiating viable cells, process program of apoptosis and epithelial The induce pro-inflammation by signals was confirmed by the present study that showed heterophils which responsible to cytokines as well as mononuclear cells that like to primary macrophages in mammalians and that represent good prognosis for immunity status at 3 hours post inoculation

Table1 Result of ANOVA test illustrate the effect of time 3 hours on invasion into cytosol of epithelial cells with time from different hours after 3 hours.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	16247.958	3	5415.986	21.974	.000
Within Groups	8872.962	36	246.471		
Total	25120.920	39			

The significant at 0.05 p value

on epithelial cells at 3hours and that diminished by time interval until decrease or disappear during 12 hours according to ANOVA statistical test (data not shown).several factors may contribute to the variation in comparison with that (Haider, et al., 2012) like effective dose, breed, age and virulence of the strain. The epithelial cell cycle differed significantly from that altered by salmonella because its effects compressed physiological maintenances. These competent of salmonella can modify the normality of parameters of the cell cycle (viable cells phase, apoptosis process phase, and dead epithelial cells). Converge at some point to activate a common genetically controlled apoptotic program. The gene and gene products responsible for apoptosis are presumed to be constitutively expressed in all cells and are tightly regulated products of other genes. The current study observed the potential effect death, or it may be pathologic and deleterious to the host. Although apoptosis may be initiated by many different extrinsic and intrinsic signals, these signals pathways death. Apoptosis may be physiologic and represent a mechanism with a balance between cell growth and cell

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Table ….2 *Multiple Comparisons between dependent variable (3 hours) and viable cells, apoptotic cells and irreversible dead cells.*

(I) AvPIgroup	(J) AvPIgroup	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
live **epith cells	live apoptotic epith cells	49.67000*	7.02099	.000	35.4308	63.9092
	dead apoptotic epith cells	46.05000*	7.02099	.000	31.8108	60.2892
	dead epith cells - irreversible	20.88000*	7.02099	.005	6.6408	35.1192
live apoptotic epith cells	live epith cells	-49.67000*	7.02099	.000	-63.9092	-35.4308
	dead apoptotic epith cells	-3.62000	7.02099	.609	-17.8592	10.6192
	dead epith cells - irreversible	-28.79000*	7.02099	.000	-43.0292	-14.5508
dead apoptotic epith cells	live epith cells	-46.05000*	7.02099	.000	-60.2892	-31.8108
	live apoptotic epith cells	3.62000	7.02099	.609	-10.6192	17.8592
	dead epith cells - irreversable	-25.17000*	7.02099	.001	-39.4092	-10.9308
dead epith cells _irreversible	live epith cells	-20.88000*	7.02099	.005	-35.1192	-6.6408
	live apoptotic epith cells	28.79000*	7.02099	.000	14.5508	43.0292
	dead apoptotic epith cells	25.17000*	7.02099	.001	10.9308	39.4092

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

N; B, viable cells = Live epith cells. Live apoptotic cells and dead apoptotic cells= apoptosis phase.