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The Pathological Changes Associated with The Genus of *Streptococcus* Spp. in Immune Modified Model of Rats

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Abstract

The aims of this study determined to investigate the pathological changes induced by *streptococcus pyogen* associated with modified immune model of rats, twenty different samples of urine and feces were collected from patients infected with Streptococcus pyogenes in local hospitals of Salaheddin and Kirkuk, after conformation the samples were then followed by Vitek2 analysis. The results was showing that 98% of the samples were Streptococcus pyogenes bacteria, After confirmation, the samples were then taken to the central laboratories of Tikrit University to determine challenge dose of infection in the rats, The animal groups divided into 4 groups, each group :having 5 animals, and the fourth group was negative control group, as follows The first , second and third groups injected 0.5 ml of *Streptococcus pyogenes* challenge dose. After 3 days, the first group that was injected with 0.5 was killed. As for the second group, after 24 hours they had a strong allergic reaction with itching in all parts of the body. However, movement rate of third group was affected. In conclusion, this study provides an understanding of the pathophysiology and importance of S. pyogenes infection, information that will be useful for future investigations and treatments aimed at reducing the negative effects of this bacterium on human health.

Keywords:
Immune-modified, Pathological changes, Rat model, *S. pyogenes*

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Introduction

For research into the pathogenesis of diseases caused by *Streptococcus pyogenes*, the groundbreaking work of the labs of Joseph Ferretti, Patrick Cleary and June Scott (Ji et al., 1996; Perez-Casal et al., 1993; Simon and Ferretti, 1991**)** established a methodology for the manipulation of the *S. pyogenes* genome, and opened the door for the analysis of pathogenesis that followed the principles elaborated by Falkow. This work spurred the development of new in vivo models that could be used to investigate the role of specific virulence factors in *S. pyogenes* pathogenesis. However, for *S. pyogenes*, the development of in vivo models has proven to be challenging for a number of reasons: First, *S. pyogenes* is a strictly human pathogen and is exquisitely adapted to its human host to the extent that many of its important virulence factors (for example, its several secreted superantigens and its plasminogen activator streptokinase (Kasper, et al., 2014; Reglinski and Sriskandan, 2014; Sun, et al., 2004;) only have activity against humans cells and proteins. The second issue reflects *S. pyogenes* remarkable versatility as a pathogen, as it may cause diseases that result from very different pathogenic mechanisms. Most of these fall into one of three broad classes (Cunningham, 2000; Cunningham, 2012; Ralph and Carapetis, 2013; Reglinski and Sriskandan, 2014; Wong and Stevens, 2013): first, local, lesional diseases in soft tissue characterized by inflammation, which can result in considerable damage to tissue in more severe manifestations; second, both local and systemic diseases that arise from damage caused by secreted streptococcal toxins; and third, immune dysfunction that results from an inappropriate immune response to streptococcal antigens. The third challenge to model development arises from the range of different tissue compartments that *S. pyogenes* can damage, which ranges from skin and soft tissue to internal organs like the heart and kidneys and to any number of different sites in the skin and other soft tissues. A final major challenge to model development is the population of *S. pyogenes* itself, which has proven to have extensive strain diversity despite its restriction to a human habitat (Bessen, 2009). This means that there is no single strain of *S. pyogenes* that can be considered representative of the population as a whole and, that relatively few strains have been shown to be virulent in any given animal model.

Despite these challenges, the previous two decades have seen the development of an impressive number of in vivo models in a diversity of animal species, ranging from invertebrates to primates, that have proven useful in the dissection of *S. pyogenes* gene/pathogenesis relationships (Chhatwal and Graham, 2017). In considering these models, it is important to note that there is no single comprehensive model of *S. pyogenes* infection. In fact, there is no single model that can accurately reproduce the authentic pathogenesis of any specific *S. pyogenes* disease. Instead, various models have been developed to model different aspects of various pathogenic mechanisms, and as a result, a thorough understanding of any particular model's strengths and weaknesses is an important consideration for experimental design, for interpretation of results as they apply to

understanding pathogenesis in that model system, and for extrapolation to the mechanism by which any S. *pyogenes* gene may contribute to human disease. In the following sections, we will review the salient features of the animal models that have proven particularly useful in modern analyses of *S. pyogenes* (Bessen et al., 2005).

Material and methods

Ethical approval

All methods were followed in ethical dealing with animals, according to the instructions of the Ministry of Higher Education and Scientific Research in Iraq.

Culture Media:

All media were prepared according to manufacturing company instructions after they were heating on a benzene burner, dissolve the constituents completely and then autoclaved, they were sterilized at 121C (15 Ib/inch2) for 15 min. Then the media were incubated at 37C for 24 hours to ensure sterilization, contaminated media were discarded. The media identification of Streptococcus pyogenes: was done by using the following media:

1. Blood agar

It was prepared according to supplied company instruction and sterilized by autoclaving at 121°C/15 pound/inch2 for 15mins, the medium was cooled to 45-50oC and then 5% as a final concentration of human blood was added. Then gently mixed and poured into sterile Petri dishes. This medium was used for the isolation, cultivation as well as detection the blood hemolysis ability of bacterial isolates (Prescott and Harley, 2002)

2. MacConkey agar (Fig. 1)

It has been prepared according to the manufacturing company (Himedia /India). It was prepared by adding 51.5 g of base medium to 1000 ml D.W. This medium contains the crystal violet to prevent grow the Gram positive bacteria and allow to grow the Gram negative bacteria (Govan & Deretic ,1996).

Isolation and Identification of Bacteria

Bacteria isolated on MacConkey agar, blood agar, then bacterial isolates were examined and identified by cultural, microscopic, biochemical tests, and the Vitek2 system. The appearance of colonies on the MacConkey agar, and Blood agar was studied with respect to their shape, color, and other characteristics (Juariah, et al., 2019).

The Vitek 2 analyzer for routine bacterial identification (Fig. 2)

The identification of the Streptococcus pyogenes by Vitek 2 Gram-Positive analyzer diagnosis of Streptococcus pyogenes. The VITEK® 2 GP identification card (GP) is intended for use with VITEK® 2 Systems for the automated identification of most significant Gram-positive bacteria is based on 64 biochemical tests measuring carbon source utilization, inhibition and resistance, and enzymatic activities. Identification results are available in approximately 8 h or less. Briefly, Streptococcus pyogenes was cultured on blood agar for 18–24 h at 37°C before subjected analysis.

Fig. 1. Phenotypic appearance of *Streptococcus* bacteria colonies on several diagnostic media showing pink colonies on MacConkey agar medium, and Milky white, non-hemolytic colonies on blood agar medium.

bioMérieux Customer: Microbiology Chart Report Printed November 12, 2023 11:14:52 AM CST

Patient Name: Patient ID:
Location: Physician:
Lab ID: Sarmad Isolate Number: 1

Organism Quantity:
Selected Organism : Streptococcus pyogenes
Source: Collected:

Comments:	

Identification Information	Analysis Time:	4.82 hours	Status:	Final
Selected Organism	98% Probability	Streptococcus pyogenes		
	Bionumber:	051412364711271		
ID Analysis Messages				

Biochemical Details																	
2	AMY	-	4	PIPLC	-	5	dXYL	-	8	ADH1	+	9	BGAL	-	11	AGLU	+
13	APPA	+	14	CDEX	-	15	AspA	-	16	BGAR	-	17	AMAN	-	19	PHOS	+
20	LeuA	+	23	ProA	-	24	BGURr	-	25	AGAL	-	26	PyrA	+	27	BGUR	-
28	AlaA	+	29	TyrA	+	30	dSOR	-	31	URE	-	32	POLYB	+	37	dGAL	+
38	dRIB	-	39	lLATk	-	42	LAC	+	44	NAG	+	45	dMAL	+	46	BACI	+
47	NOVO	+	50	NC6.5	-	52	dMAN	-	53	dMNE	+	54	MBdG	-	56	PUL	-
57	dRAF	-	58	O129R	+	59	SAL	-	60	SAC	+	62	dTRE	+	63	ADH2s	+
64	OPTO	+															

Fig. 2. *Streptococcus pyogenes* (98%) excellent identification by VITEK 2 GP card.

Microscopic Examination

Bacterial isolates were examined for Gram stain, shape, and arrangement was observed using the light microscope (Spellerberg, & Brandt, 2022).

Experimental Animals:

Twenty female albino white rats, with an average age of 10 to 12 weeks were used in this study. They kept in separated clean and disinfected cages fed commercial pellets and tap water, rats were kept for 30 days for adaptation before the treatment.

Experimental Design for study:

Twenty females' white rats were divided randomly into 4 groups and treated as follows:

1. 1st group was administered 0.5 ml *Streptococcus pyogenes* challenge dose, I/P 3 day I/P.

2. 2nd group was inoculated with 0.5 ml of *Streptococcus pyogenes* challenge dose, S/C. 3day I/P.

3. 3rd group was immunized with 0.5 S/C challenge dose, S/C. 3day 1/P.

4. 4th group, control negative group was given 0.5 ml s/c normal saline I/P.

Histopathological Examination:

The tissues were fixed with a (10%) tampon formaldehyde solution. Immediately after removal, the specimens of 1 x 1 x 1 cm dimensions including the spleen, liver, lung, heart and brain. The specimens were washed with tap water after 72 hours of fixation, and then processing took place regularly by upgrading the alcoholic concentration from 70% to 100% in every single hour to removed water, by extracting xylol from water and by infiltrating the samples with a 58 °C semi-liquid paraffin wax, then by covering the tissue with the specimens Hematoxylin and Eosin (H & E) darkened all tissues and histopathological changes were seen under a light microscope (Luna, 1968).

Determination of *Streptococcus pyogenes* challenge dose

Streptococcus pyogenes cultured on blood agar then incubated at 37 °C for3day. Two rats were inoculated I/P with 0.5 ml of bacterial suspension growth, the animals were sacrificed at 24 hrs post inoculated. and pieces from internal organs were cultured on the blood agar for 24 hrs at 37 °C. This process was recurrent until the inoculated animals died within 24 hrs. 12 rats both sexes were divided into three equal groups and they were inoculated with 0.5 ml of bacterial suspension containing $1x10^5$, $1x10^6$, and $1x10^7$ CFU of virulent *Streptococcus pyogenes* respectively. We recorded the number of the dead animal during 24-48 hrs post-inoculation. Preparation of the bacterial suspension for the counting was made according to Miles method (Miles et al., 1938).

Results

Histopathological Examination

The current results for animal died during 72 hrs post-infection shows histopathological changes in the lung, kidney, and liver tissues.

Lung: (Fig. 3)

Histological section in the lung of rat 72hr post-infection with *Streptococcus pyogenes* with $1X10^5$ CFU/ml shows congestion with inflammatory exudate in interstitial tissue with infiltration of the inflammatory cell (II) and ulcer performed (H & E stain at 10, 40 X). While, histological Section in the lung 72 hrs post-infection with *Streptococcus pyogenes* with $1X10^6$ CFU/mL show hemorrhage, congestion, and edema in with thickening of interalveolar septa.

Histological Section in the lung 72 hrs post-infection with *Streptococcus pyogenes* with $1X10^7$ CFU/ml show mild congestion

and edema together with H & E stain as shown in Fig. 3.

Liver: (Fig. 4)

Histological Section in the liver of Rat 72 hrs post-infection with *Streptococcus pyogenes* with 1 X 10^5 CFU/ml shows many areas of congestion thrombosis in portal area, B shows inflammatory cell infiltration in portal area, appears necrosis cell (coagulative necrosis).

Histological Section in the liver of Rat 72 hrs post-infection with *Streptococcus pyogenes* with 1 X 10^6 CFU/ml shows congestion with necrotic cell.

Histological Section in the liver of Rat 72 hrs post-infection with *Streptococcus pyogenes* with 1 X 10^7 CFU/ml shows dilated sinusoid and engorgement with inflammatory cell. (H & E stain) as shown in Fig. 4.

Kidneys: (Fig. 5)

The histological sections show nearly normal cortex and medulla of the kidney without clear lesion. Histological Section in the kidney of rat 72 hrs post-infection with *Streptococcus pyogenes* with 1 X 10^5 CFU/ml shows many areas of degenerative changes in the distal and proximal tubules.

Multiple dilated cortical tubules are lined by epithelial cells that are hyper-eosinophilic, shrunken, and pyknotic indicative of necrosis, the glomerular tuft appear shrinkage.

Histological Section in the kidney of rat 72 hrs post-infection with *Streptococcus pyogenes* with 1 X 10^6 CFU/ml shows congestion.

Histological Section in the kidney of Rat 72 hrs post-infection with *Streptococcus pyogenes* with 1 X 10^7 CFU/ml shows congestion and atrophy of glomeruli (H & E stain).

Discussion

The recent results histopathological of groups 1, 2, and 3 post-infections with challenge dose *Streptococcus* with challenge dose 1 X $10^{5,}$ 1 X $10^{6,}$ and 1 X 10^7 respectively show many pathological lesions in the intestine as well as congestion and necrosis with many aggregations of different types of inflammatory cells and necrosis in villi of mucosa and ulcer in the submucosa.

This is because the bacteria used in the current experiment were isolated from infected people, which means that they possess strong virulence factors that enable them to cause multiple pathological lesions in the body and resist the body's immune system. Furthermore, this evidence was supported idea that mentioned by the present study showed a severe pathological lesion in the internal organs of control positive group and these results may indicate that *Streptococcus pyogenes* overcome innate immune responses of the host and it was supported by evidence recorded by previous reports (Shulman et al., 2012).

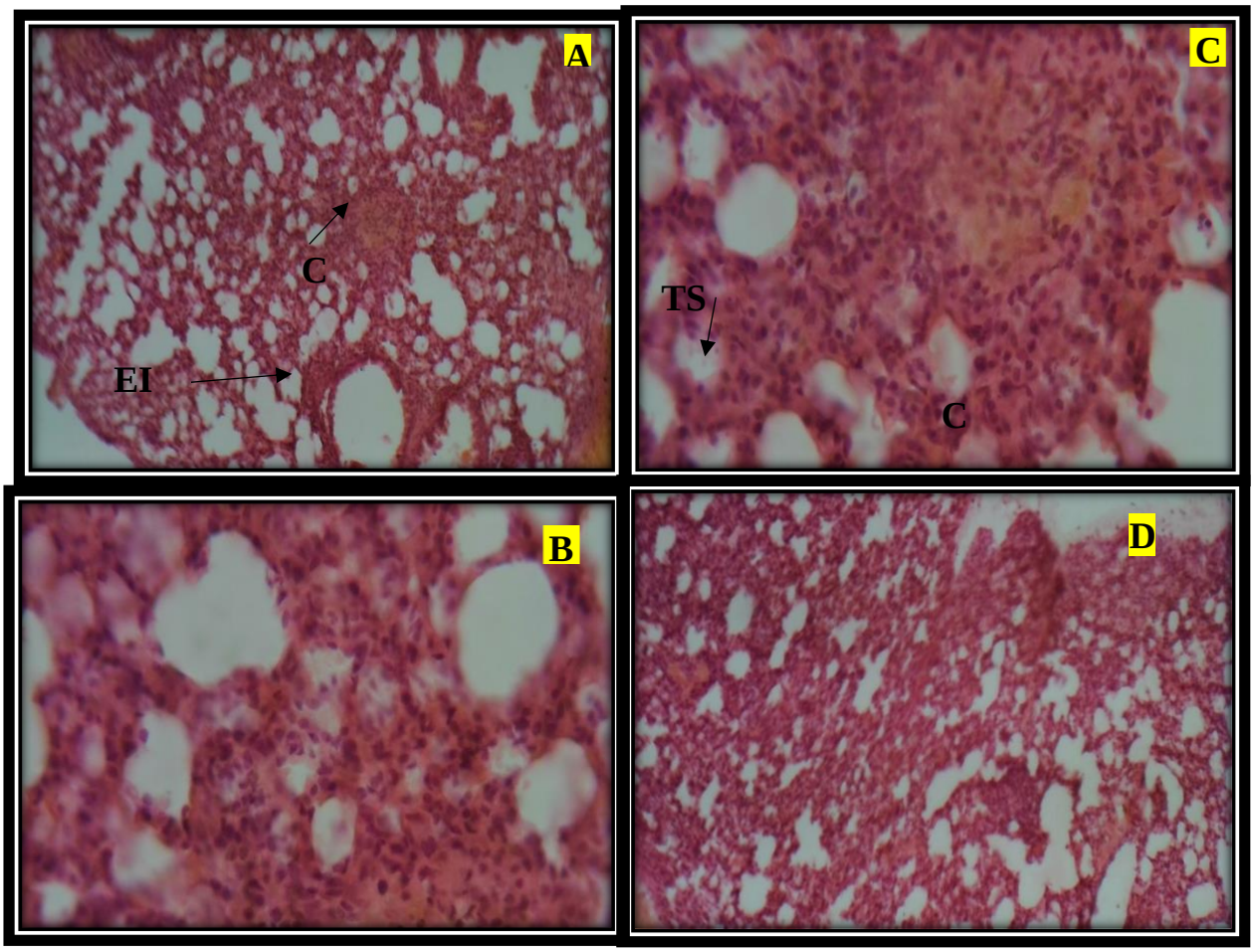

Fig. 3. (**A-B)** Histological Section in the lung of rat 72 hrs post-infection with *Streptococcus pyogenes* with 1 X 10^5 CFU/ml shows Congestion (CO) with inflammatory exudate (EI) in in interstitial tissue with infiltration of the inflammatory cell (II) and ulcer performed (U) (H & E stain at 10, 40 X). Histological Section in the lung **(C)** 72 hrs post-infection with *Streptococcus pyogenes* with 1X10^6 CFU/mL show hemorrhage, congestion (CO), and edema (E) in with thickening of interalveolar septa (TS). Histological Section in the lung **(D)** 72 hrs post-infection with *Streptococcus pyogenes* with 1X10^7 CFU/ml show mild congestion and edema **(E)** together with (H & E stain at 10 X).

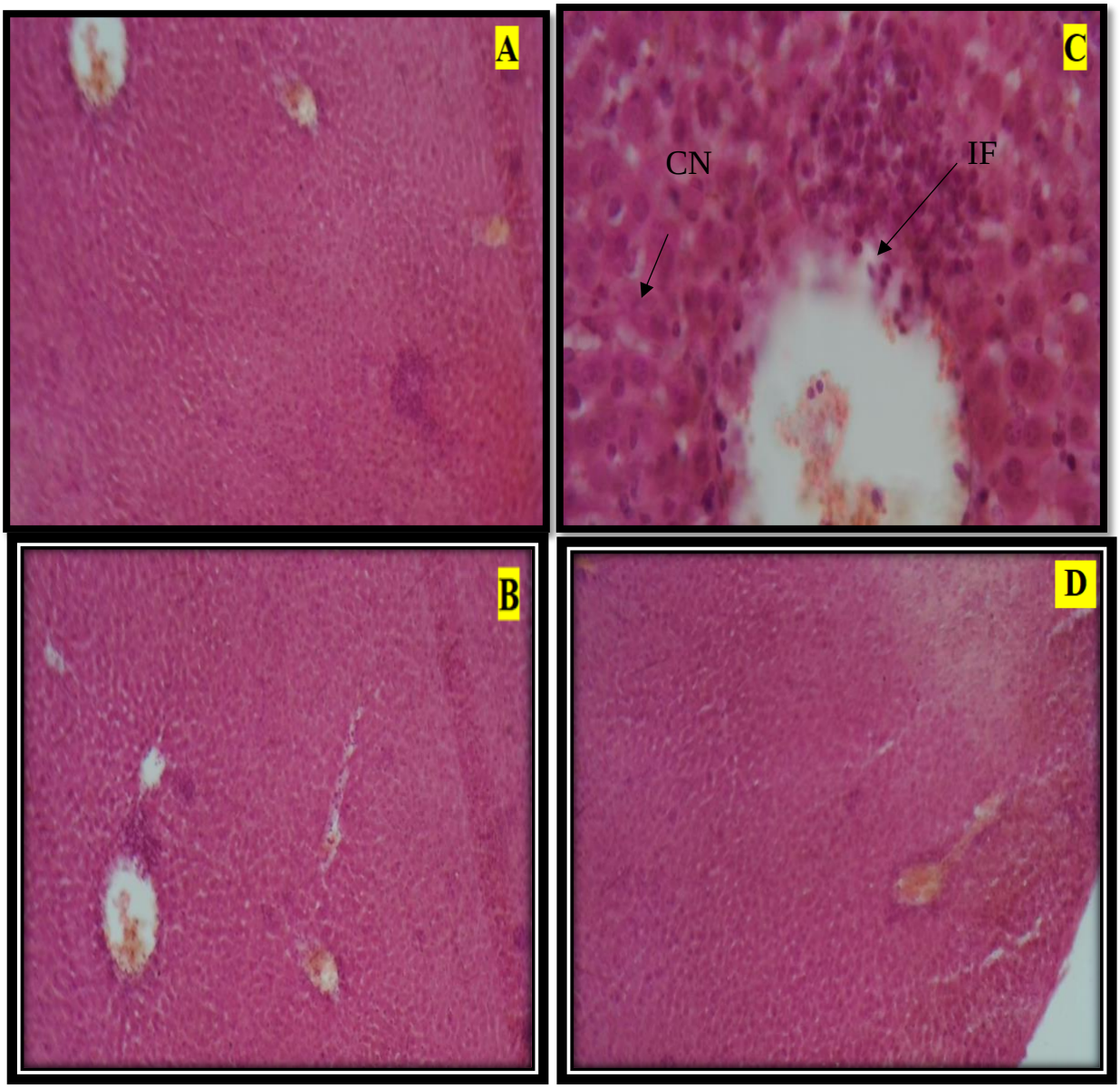

Fig. 4. (A) Histological Section in the liver of rat 72 hrs post-infection with *Streptococcus pyogenes* with 1 X 10^5 CFU/ml shows many areas of congestion thrombosis in portal area, (**B)** shows inflammatory cell infiltration in portal area (IF), in C appears necrosis cell (coagulative necrosis) (CN). (**C)** Histological Section in the liver of rat 72 hrs post-infection with *Streptococcus pyogenes* with 1 X 10^6 CFU/ml shows congestion (CO) with necrotic cell (N). (**D)** Histological Section in the liver of rat 72 hrs post-infection with *Streptococcus pyogenes* with 1X10^7 CFU/ml shows dilated sinusoid and engorgement with inflammatory cell. (H & E stain at 10, 20 and 50 X).

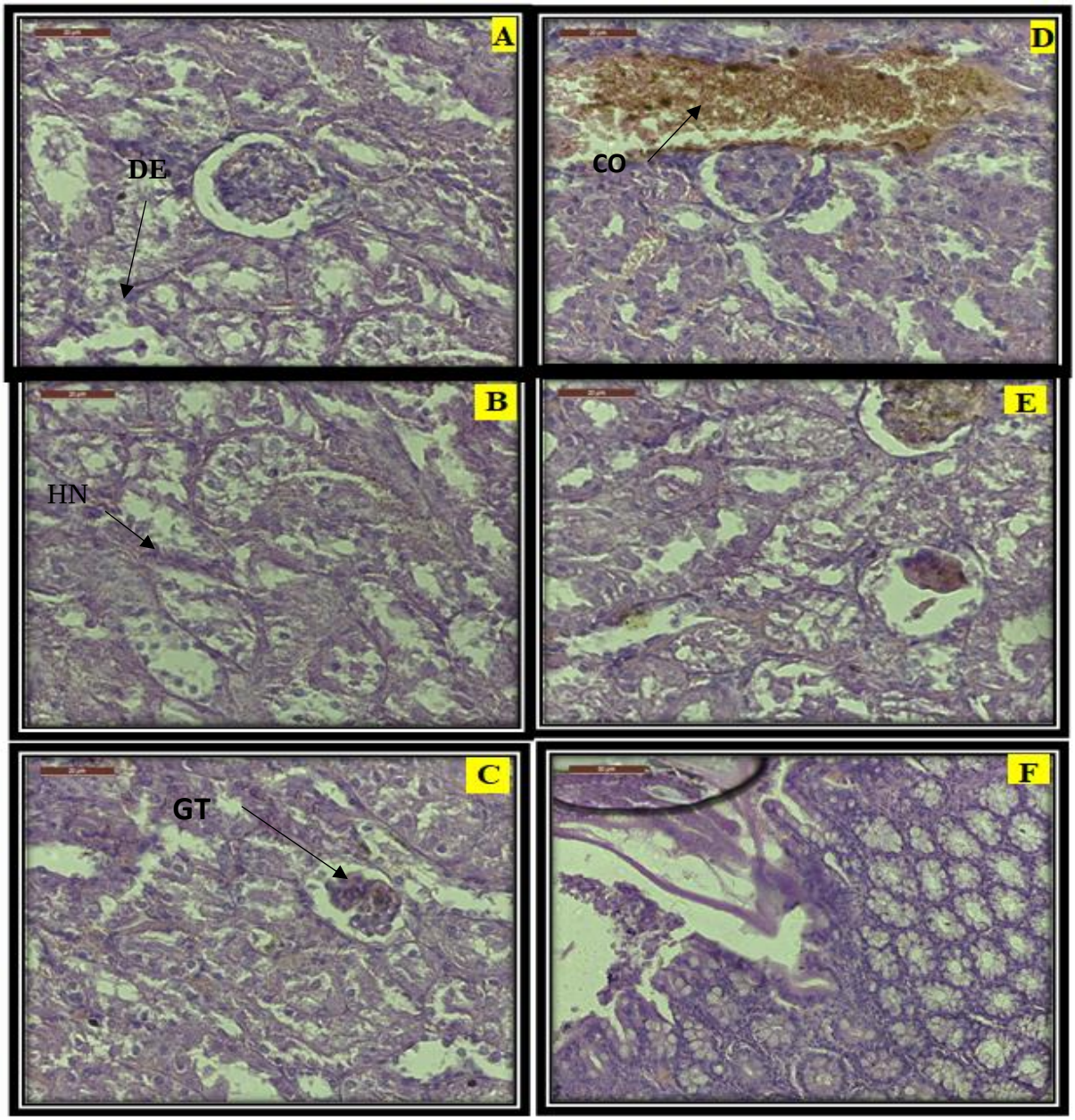

Fig. 5. Nearly normal cortex and medulla of the kidney without clear lesion. (A) Histological Section in the kidney of Rat 72hr post-infection with *Streptococcus pyogenes* with $1X10^5$ CFU/ml shows many areas of degenerative changes in the distal and proximal tubules (DE), **(B)** Multiple dilated cortical tubules are lined by epithelial cells that are hyper-eosinophilic, shrunken, and pyknotic indicative of necrosis (HE). **(C)** The glomerular tuft appears shrinkage (GT). **(D)** Histological Section in the kidney of rat 72 hrs post-infection with *Streptococcus pyogenes* with $1 X 10^6$ CFU/ml shows congestion (CO). **(E)** Histological Section in the kidney of rat 72 hrs post-infection with *Streptococcus pyogenes* with $1 X 10^7$ CFU/ml shows congestion (CO) and atrophy of glomeruli (AG). (H & E stain at 10, 20 and 50 X).

The throat and skin of the human host are the main reservoirs for *S. pyogenes* (Johansson et al., 2010). Which can produce superficial impetigo or deep cellulitis but also more serious infections such as sepsis, necrotizing fasciitis and streptococcal toxic shock syndrome (Hirose et al., 2021; Walker et al., 2014). To successfully colonize or establish infection in the skin, pathogens must possess virulence determinants to evade these immune factors and acquire nutrients, the availability of which may be restricted by the host under the concept of trophic immunity. Elucidating the bacterial metabolic pathways that are essential for survival in vivo could reveal unique targets for new therapeutics (Hirose et al., 2021).

The severe pathological lesions in the examined organs of non-immunized infected animals (all groups) in the present study, may indicate that the bacterial strain used in this study was highly virulent, and overcame the host immune response this led to the dissemination to all internal organs causing multiorgan failure and death the infected animals, this idea is in agreement with Camejo et al., (2011), who recorded that the listeria possesses unique virulence factors to invade host, evade immune cells and to cause infection. Also, the widespread of infection in the present study may indicate this pathogen may cause the depressed immune system of the hosts, this evidence was in consistent with Carrero et al., (2006), who found that *S. pyogenes* can induce inhibit the innate immune response through stimulated production of type I interferon's that induce Tcell apoptosis during early infection in addition to, this pathogen can stimulate phagocytic cells to, greater secretion of IL-10, also *S. pyogenes* can escape the phagolysosome into cytosol of target cells via Induction of type I interferon (McCaffrey et al., 2004), so highly virulence *S. pyogenes* can destroy immune cells of the host such as macrophages, DC and T lymphocytes by the extracellular release of particularly in the liver and spleen that lead to the death of lymphocyte and hepatocytes (Vilchis et al., 2019; Strus et al., 2017).

We established a model of *Streptococcus pyogenes* I/P infection in albino Rats, the current study demonstrated the high prevalence of *S. pyogenes* in rat. Experimental infection using a selected strain confirmed its ability to induce pyogenes with ulcers, liver necrosis, and nephritis with many pathological changes. Infection caused low feed consumption, average body weight, weekly body weight gain, and FCR. Measures are needed to control *S. pyogenes* infection in human and animal farms by using suitable probiotics to avoid and overcome multidrug resistance to reduce economic losses, and in conclusion, our study shows the critical importance of pulmonary infection pneumococcal virulence, disease severity, and pulmonary inflammation in immune modified rats.

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