

Immunopathological Effects of Streptococcus Pyogenes Isolated from Human and Sheep Throat Swabs: Experimental Infection in Male Albino Rats

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ABSTRACT

Background: Streptococcus pyogenes is a significant pathogen in humans and animals, known for its invasive potential and immunopathological effects. This study aimed to investigate the immunological and pathological impact of *S. pyogenes* isolated from human and sheep throat swabs, focusing on immune markers and histopathological changes. **Objectives:** The study evaluated the levels of Immunoglobulin G (IgG), Interleukin-10 (IL-10), and Tumour Necrosis Factor-alpha (TNF- α) in conjunction with histopathological alterations in the brain, lungs, and kidneys following *S. pyogenes* infection. **Methods:** Throat swabs (n=100) were collected from sheep and humans presenting with respiratory symptoms between January and May 2022. Conventional bacteriological methods confirmed the presence of *S. pyogenes* isolates. Male albino rats (n=20), aged 8–12 weeks and weighing 200–250 g, were acclimatised for 4 weeks and divided into two groups: Group 1 served as a negative control, receiving phosphate-buffered saline, while Group 2 received 0.5 mL of *S. pyogenes* suspension (1×10^7 CFU/mL) intraperitoneally. Rats were monitored for clinical signs, and subsets were sacrificed at 7- and 14-days post-infection. Blood samples were analysed for IgG, IL-10, and TNF- α levels, while tissue samples (brain, lungs, kidneys) were processed for bacterial isolation and histopathological examination. Statistical analysis was conducted using ANOVA and the least significant difference (LSD) test ($p \leq 0.05$). **Results:** *S. pyogenes* was identified by its β -hemolytic colonies on blood agar and characteristic inhibition zones on bacitracin susceptibility tests. Immunological analysis revealed significantly higher IgG titers at 14 days (32.39 ± 0.87) compared to 7 days (12.22 ± 0.23) and negative controls. IL-10 and TNF- α levels showed a significant increase at 7 days (372.83 ± 14.96 and 364.79 ± 4.91 , respectively) and peaked at 14 days (769.21 ± 18.73 and 468.29 ± 4.21) compared to controls (374.03 ± 14.31 and 370.79 ± 2.36 , respectively). Postmortem findings included multiple pneumonic consolidations, abscess foci in lung tissues, and histopathological evidence of abscess formation, granulomatous-like lesions in the lungs, cortical cystic changes in the kidneys, and neuronal nuclear pyknosis with perineuronal oedema in brain tissues. **Conclusion:** *S. pyogenes* infection in male albino rats induces significant immunological and pathological changes, characterized by elevated IgG, IL-10, and TNF- α levels and extensive tissue damage in the brain, lungs, and kidneys. These findings highlight the pathogen's invasive nature and its impact on host immunity, emphasizing the need for targeted therapeutic strategies to mitigate its effects.

Keywords: Biochemical tests, Cytokines, IgG, Histopathological, *S. pyogenes*, Throat Swab.

INTRODUCTION

Gram-positive bacteria [1] are bacteria that retain thick peptidoglycan cell walls and classified depending on their blue color when stained by gram stain and comprised as cocci, bacilli, or branching filaments, some of these bacteria grow in clusters (Staphylococcus) [2] and others in chains (Streptococcus), Streptococcus subdivide into Streptococcus pyogenes (Group A), Streptococcus agalactiae (Group B), enterococci (Group D), Streptococcus viridans, and Streptococcus pneumonia [3, 4]. Streptococcus pyogenes is a gram-positive, B-hemolytic, facultative aerobic and capsulated, can cause different types of infections like pyogenic, toxigenic and immunologic infections and required highly coordinated host response for bacterial intracellular killing through neutrophil and macrophage [5, 6], because it's starting their infection with exotoxin that interact with host cells via specific receptors on cell surface like Toll-like receptors [7], also play major role in causing sepsis (multi-steps process and potentially life-threatening un-control inflammatory response induced by abnormal regulation of host cells toward the infection result in multi-organs failure ended by death) [8] because these bacteria have ability to expression pro-inflammatory toxins and many virulence factors which have effective role on tissues,

cells and immune response components for that enable them to escape the immune defenses and disseminate to remote organs [9], therefore; infected host show variable pathophysiological mechanism and immune response to sepsis depending on bacterial type and initiated factors for exotoxin production [10]. During systemic inflammatory response syndrome (SIRS), the immune system response is a complex dynamic and time-dependent process affected by mis regulation, leading to an imbalance response; the innate immune system cells release high levels of pro-inflammatory cytokines triggering a "cytokine storm", and sometimes induce apoptosis of immune cells during the first hours of sepsis [11]. pro-inflammatory cytokines have an important role in host protection from invading pathogens during the initial stages of infection, sepsis development is induced by an imbalance between the pro- and anti-inflammatory mechanisms in the host, another process can occur simultaneously to SIRS is a compensatory anti-inflammatory response (CARS) result from hyper-inflammatory process and characterized by increased anti-inflammatory and decreased pro-inflammatory cytokine production [12], also the exacerbated hyper-inflammatory response can cause damage in uninfected tissue leading to dysfunction of different organs

and systemically inducing hypotension, vasculature damage and clotting events, which finally by death [13].

MATERIALS AND METHODS

Ethical Approval

The local Committee for Animal Care and Use at the College of Veterinary Medicine, University of Baghdad, reviewed and approved all procedures involved in the current study (Approval Number: 380/P.G. dated February 19, 2024).

Animals and Housing

Twenty male albino rats were used in the current experimental age (8-12) weeks, and weights ranged between (200-250) g. The breeding and maintenance of rats were conducted at the Animal House, Veterinary Medicine College, Baghdad University. Before the initiation of the experiment, acclimating of rats for 4 weeks in standard cages (20×30×50 cm³) with 5 animals per cage under controlled environmental conditions of (22 ± 3 °C), (50 ± 5 %) relative humidity, and 14/10-hour light/dark cycle. Available water and standard feed pellets were ad libitum throughout the experimental period. Twenty rats were divided into two groups: the negative control group received phosphate-buffered saline (PBS). In contrast, the positive control group was infected intraperitoneally (I.p.) with 0.5 mL of a bacterial suspension containing 1×10^7 CFU/mL of *S. pyogenes*.

Sample Collection

A total of 100 throat swabs were collected from sheep and humans suffering respiratory signs such as nasal discharge, sneezing, anorexia, restlessness, depression, and fever from different hospitals and clinics such as Abu-Ghraib (white gold village), Collage of Veterinary Medicine Field, Veterinary clinic in Al-Suwayrah, Al-Suwayrah hospital, Al-Razi center, Central Teaching Hospital of Pediatrics, Medical city, the collection occurred randomly under sterilized conditions throughout the period from January to May 2022, by using sterile swabs with media then labeled and transported to the pathology laboratory, College of Veterinary Medicine/University of Baghdad, to identify *Streptococcus pyogenes* by different immunological and biochemical tests.

Preparation of Cultural Media

The culture media used are brain heart infusion broth, brain heart infusion agar, blood agar, and selective media agar (Himedia). All media were prepared according to the manufacturers' instructions, then autoclaved at 121°C and 15 pounds per square inch for 15 minutes. After that, they were distributed into Petri dishes and test tubes then incubated at 37°C for 24 hours (Ahmed *et al.*, 2018).

Identification of Streptococcus Pyogenes

The suspected bacterial colonies were identified as *S. pyogenes* using standard microbiological techniques, including *B-Hemolysis* on blood agar plates, catalase and oxidase tests, sensitivity to bacitracin discs, and the morphological appearance of Gram-positive cocci (Streptococci) under a light microscope. Further confirmation was obtained by using Group A streptococcus selection media.

Bacterial Isolation and Culturing

For isolated bacteria, a loopful of suspected swabs was streaked on a blood agar plate and incubated at 37 °C for 24 hours. under (5-10%) CO₂ condition [14, 15]. Based on morphology of the colony on the

above media, when staining by gram's stain, in addition to another frequently biochemical tests like disc diffusion test [16], that used to determine the susceptibility of isolated bacteria toward bacitracin and finally, helps in recognized group A *Streptococcus pyogenes* (GAS) from other types of *B*-hemolytic pathogens [17] All of the above gave positive results for the isolated and initially identified Streptococcus pyogenes.

Viable Cell Count of Bacterial Suspension

Tenfold dilutions of the original bacterial suspension were performed by transferring 1 ml of bacterial suspension to the first tube containing 9 ml of PBS, then 1 ml from the first tube transferred to the second tube, and so on until tube number 10. This resulted in dilutions ranging from 10⁻¹ to 10⁻¹⁰. A viable cell count was conducted by pipetting 0.1 mL from the chosen dilution series, which was then poured over the middle of the agar plate's surface. Then, the sample was spread evenly over the agar's surface with careful rotation underneath the Petri dish at a 45-degree angle, and the plates were incubated for 24 hours at 37 °C. The dilution that gave a countable number of colonies in the corresponding plate was chosen to calculate the bacterial concentration in the original bacterial suspension according to the following formula:

$$\text{CFU/ml} = \frac{\text{No. of colonies} \times \text{Total dilution factor}}{\text{Volume of culture plated in ml}}$$

Estimation of Infectious Dose

The infectious dose was estimated using the Reed-Muench method (1938). Twenty-four male rats were divided into six groups (n = 4) at random. The first three groups were inoculated intraperitoneally with 1 ml of dilutions (10⁶, 10⁷, and 10⁸) CFU/ml, and the remaining three groups were inoculated with 0.5 ml from the same dilutions mentioned above, respectively. All groups were monitored for 10 days to notice the symptoms. The seventh dilution, 0.5×10^7 CFU/ml, showed clear clinical signs in rats, and this dose was later used as a challenge dose (Michailova *et al.*, 2000).

Blood Sample Collection

About (4 mL) of blood samples were collected at the end of the experiment from all animals after chloroform inhalation anesthesia (CDH, Italy) via cardiac puncture using a disposable syringe of 5 ml. Then, the collected blood was placed in plain gel tubes, left at room temperature for 10-20 minutes for clotting, and centrifuged at 3500 rpm for 5 minutes. The serum was liquefied into labelled Eppendorf tubes (Karl, China) and kept frozen at -20°C until used for immunological analysis (Abbasa *et al.*, 2023).

Histopathological Examination

After euthanized rats by using an overdose of anesthesia, tissues from the brain, lung, and kidney were taken and fixed in phosphate-buffered formalin (10%) for (2-3) days. Following fixation, samples were sectioned to 0.5 cm thickness and put in plastic cassettes [18]. Tissue dehydration and clearing occur automatically using the Italian tissue processor Histo-Line Laboratories ATP1000. Subsequently, the dehydrated tissues were embedded in paraffin wax using a Histo-Line Laboratories HESTION TEC2900 embedding system, which regulated the temperature with a TEC2900 Thermal Console (Histo-Line Laboratories, Italy). The section tissue blocks at thickening between (4-5) µm by using a Histo-Line Laboratories MRS3500 rotary

microtome (Italy) [19] They floated the tissue sections in a water bath at 37°C and on a temperature-controlled hot plate, both regulated by the TEC2900 Thermal Console, before mounting them on glass slides. Finally, staining by routine Hematoxylin and Eosin stains (HandE, Dakocytomation, Denmark) [20] and examined under a light microscope (Olympus, Japan) at 40× and 10× magnifications for detailed histological assessment [21].

Statistical analysis

The SAS program was used to analyze the data and find the influence of various factors on the study's parameters. The Least Significant Difference (LSD) test was used to analyse variance using Analysis of Variance (ANOVA). Two ways to identify the significant differences in the results were determined at $P \leq 0.05$, according to SAS (2018).

Results

Biochemical Identification of Bacteria

Standard microbiological techniques were used in the diagnosis of *S.pyogenes* on blood agar under anaerobic conditions at 37°C for 24 hours, which appeared as a shining colony surrounded by zone of β -hemolysis with evidence of small white bacterial cocci about 0.5-1 μm in size when culturing on Group A streptococcus selective agar media as qualitative procedure for detecting the isolated bacteria. The antimicrobial sensitivity test was conducted using a bacitracin disc to distinguish *S. pyogenes* from other types of β -hemolytic bacteria (*S. agalactiae*), with an estimated clear zone of inhibition around the antibiotic disc.

Postmortem Examination

Infected albino rats appeared depressed, anorexia, tremble, and dullness during the experimental period. Estimating the prevalence of gross and histopathological lesions in the infected group was performed. No gross changes were observed in the control positive group infected with *S.pyogenes* in any organ, except for those revealed in lung tissue, characterised by multiple pneumonic consolidated foci at 7 days post-infection. Notably, obvious yellowish foci of abscess were observed at 14 days post-infection.

Measurement of Humoral and Cell Mediated Immunity by Elisa Technique

The serological finding of the 2nd positive control group at 14 days post infection recorded significant elevator in antibodies titer, TNF- α , and IL-10 (32.39 ± 0.87 , 468.29 ± 4.21 and 769.21 ± 18.73) respectively compared with the 1st negative control group as above (12.82 ± 0.59 , 370.79 ± 2.36 and 374.03 ± 14.31), while at 7 days post infection the 2nd group noticeable decline in the concentration of IgG and mention's cytokines (12.22 ± 0.23 , 364.79 ± 4.91 and 372.83 ± 14.96) consecutive compared with the 1st group (12.82 ± 0.29 , 368.79 ± 6.28 , 373.63 ± 14.19) respectively.

Histopathological Finding

The histopathological changes in lung tissue at 7 days post-infection demonstrated sever pulmonary hemorrhage with congestion and diffused Poly morphonuclear cells PMNCs infiltration accompanied with abscess formation, while renal tissue notice obvious cortical cyst formation with perivascular Mononuclear cells MNCs infiltration another section showed focal abscess formation with

interstitial hemorrhage while brain tissue revealed moderate congestion and dilation of meningeal and cerebral blood vessels with pyramidal vacuolation. The pathological manifestation of the lung at 14 days post-infection noticed focal abscess formation with thickening of interalveolar tissue and another section shows a focal aggregation of MNCs forming granuloma-like appearance in pulmonary tissue with evidence of destructive lesion in adjacent alveolar tissue, while kidney tissue show diffused interstitial MNCs infiltration with slight bowman space dilation with moderate vascular congestion and dilation the in the cerebral cortex with vacuolation of pyramidal cells in the hippocampal region accompanied with degenerated neurons in brain tissue, also show neuronal pyknosis with perineuronal edema.

Discussion

Streptococcus pyogenes is a significant global human pathogen that causes a wide range of acute infections, including respiratory tract infections, skin and soft tissue infections, induced sepsis, and dysfunction of multiple organs due to a disorder of the host immune response. In this study, several sources of gram positive bacterial infection were isolated and seem to be almost exclusively restricted to humans with limited reports of seclusion from animals and can cause symptoms arranged from mild to severe in many different species; however, asymptomatic transporters have a significant role in animal infection when consumption food contaminated by *S. pyogenes* [22], and this result accorded with [23] who explain that human is the primary infected host after that transfer to commercially farmed animals like cattle, rabbits, sheep, domestic cat and dogs and captive animals (monkeys) while another study by [24, 25] showed that animals might be a symbol for a new receiver of catalase-negative bacteria but point to the capacity of human-derived *S. pyogenes* isolates to colonize and infect animals. *Streptococcus* selection agar was used for the first time in the present study for the identification of *S. pyogenes*, providing adequate culturing conditions for the primary isolation of *Streptococcus pyogenes* from throat and upper respiratory specimens.

Also, additional biochemical tests were used bacitracin disc in presumptive distinguish of *S.pyogenes* from other types of β -hemolytic (*S.agalactia*) and non-hemolytic types of streptococcus by formation zone of inhibition around bacitracin disc indicates that the organism is susceptible/sensitive to this antibiotic and these observations like with [26] who notice large inhibited zone around the bacitracin disc because this type of antibiotic is a polypeptide produced by *Bacillus subtilis* and inhibits the synthesis of bacterial cell wall and disrupts their cell membrane. The elevation level of cytokines in the infected group comparative with control negative group and these results harmonized with [27, 28] who indicated issuing of proinflammatory cytokines such as TNF- α , IL-1, IL-8, IL-6 and anti-inflammatory cytokines like (IL-10 and IL-12) lead to accumulation of MNCs in different organs of the infected host while [13, 29] revealed the effective role of macrophage (microglia) and cytokines such as IL-12, INF- γ , and TNF- α in the formation of an abscess like structure to limit the diffusion of pyogenic bacteria from the site of the lesion. Present manifestations of pulmonary abscess were recognised mainly at 7 and 14 days post-infection, while kidney abscess was noticed at 7 days post-infection. These results indicate the active proliferation of pathogen at soft necrotic tissue and this union with [30] who explain that huge number of *Streptococcus pyogenes* found at the site of

necrotic tissue because it's the foremost causative pathogen of Necrotizing soft tissue infections (NSTIs) this may be attributed to M-protein and phosphoglycerate kinase (PGK) which registered as potential inducers for activating and deregulating of neutrophilic cell lead forming loose abscess-like structure around the bacterial growth area result to release proteolytic enzymes and other effector molecules into the rounding area. In addition, *S. pyogenes* cocci and other species of streptococcus express cytolytins (streptolysin and pneumolysin), which can destroy host cells by interacting with streptococci, anti-streptococcal immunity, and complement, potentially leading to an excessive inflammatory response [3]. A recent study by Wilde *et al.* [9] found that Group A *Streptococcus pyogenes* pathogens can develop several mechanisms to evade host protection and induce exaggerated inflammation. According to above results various forms of neuronal swelling and degeneration recorded mainly at 14 day post infection and this evidence harmonized with [31] who indicated that Streptococcus group A induced brain disorders such as inflammation of neuron and activation of microglial related with injury of brain tissue and damage blood brain barrier (BBB) caused by Group A streptococcus pyogenes ability to penetrate the brain or mediated by autoantibodies recognizing the production of central nervous system epitopes in condition known as pediatric autoimmune neuropsychiatric disorders accompanying with streptococci, which involves a possible connotation with repeated streptococcal infections, also [32, 33] showed that the TNF produced in the early phase of inflammation and plays a role in communicated between immunity and brain by enhancing leaking of BBB and promote microglia activation. A recent study revealed that an elevation in TNF- α concentration is a result of inflammatory and brain injury pathology,

leading to an increase in circulation. Blocking these cytokines causes reduced injury and inflammation by preventing the breakdown of the blood-brain barrier and improving survival. This result agrees with [34], who found that inflammation of the central nervous system occurs due to Streptococcus pyogenes infection and can be attenuated by blocking TNF. Previous research by [35, 36] has noted that the capability of S.pyogenes to cause brain and meningeal abscesses results from the pathogen invading the meningeal space or brain tissue.

CONCLUSIONS AND RECOMMENDATION

The results concluded that S.pyogenes induced pathological changes in the internal organs (brain, lung, and kidney) of infected male rats, depending on the infectious dose, and affected the immune system with a significant elevation in antibody titer (IgG), IL-10, and TNF- α , specifically at 14 days post-infection. The current study is recommended for investigating the virulence genes and biofilm of S. pyogenes. Moreover, further techniques such as immunohistochemistry, immunocytochemistry, and Polymerase chain reaction (PCR) can be used to detect S.pyogenes by utilising several markers, allowing for the study of the infection's effect on the skin and rheumatic heart disease.

Ethics statement

The local Committee for Animal Care and Use at the College of Veterinary Medicine, University of Baghdad, reviewed and approved all procedures involved in the current study (Approval Number: 380/P.G. dated February 19, 2024).

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