

Histopathology of the kidney and seroprevalence of leptospirosis in wild rats in Baghdad Province, Iraq

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Abstract

Background and Aim: Leptospirosis, caused by pathogenic leptospires, is a globally emerging infectious disease affecting both humans and animals, which act as reservoirs, with large outbreaks worldwide. The role of rats in dispersing leptospirosis was never investigated in Iraq. Because of the seriousness of the disease and the scarce data regarding this disease in Iraq, this study determines the incidence of leptospirosis in rats and its renal histopathological profile.

Materials and Methods: Of 211 captured rats, 82 apparently healthy rats were included in this study. After euthanizing, 3-5-ml blood was collected by cardiac puncture. Approximately 0.5 cm³ of the kidney was collected for routine histopathology and stained using hematoxylin and eosin (H&E) and Warthin–Starry (WS) stains. Blood smears were prepared and stained with the WS stain.

Results: All rats (100%) with different age groups were immunoglobulin G (IgG)-positive, and 90.24% of them had the IgG against leptospiral antigens in kidney tissues. The juvenile age group had higher IgG levels than other age groups. Considering sex, no significant differences in the overall results were observed. Serum concentrations of blood urea nitrogen and creatinine showed significant increments in the sub-adult and adult IgG- positive groups compared with the IgG-negative groups. No significant alterations were observed in the juvenile group. Using WS stains, 13 and 1 blood smears and 0 and 8 kidney tissues were positive for leptospires in the sub-adult and adult groups, respectively. Microscopical findings of the renal cortex and medulla in the sub-adult IgG-positive group showed hemorrhage, glomerular deterioration, tubular cell degeneration and necrosis with cast formation, periarterial edema, and focal hemorrhage with congestion of peritubular arteries. The adult IgG-positive group revealed deterioration similar to that in the sub-adult group and tended to be chronic. No leptospires were observed using H&E staining.

Conclusion: IgG-positive carrier rats refer to previously exposed or infected rats. Understanding the risk of transmitting the disease to human and animals through a carrier rat's urine is highly predicted and possible mitigation of zoonotic transmission.

Keywords: histopathology, kidney, leptospirosis, rats, serology.

Introduction

Wild rats could be found everywhere. Their mobility from sewage to our kitchen serves as a reservoir for many diseases. Rats are responsible for dispersing many diseases to humans, particularly the silent “asymptomatic” disease, and leptospirosis [1,2]. Environmental factors, including humidity and flooding, play a crucial role in the spread of leptospirosis, leading to a higher incidence of this disease in tropical countries. However, because rats are ubiquitous, leptospirosis remains a public health issue even in large cities of both developed and developing countries [3,4]. Rats and most animals become infected when exposed to the urine of cohabiting infected rats

that carry different serovars. After initial infection, animals act as carriers and continue shedding bacteria into the environment [2,5]. Known risk factors include occupational exposure among farmers, butchers, and sewage workers and swimming in ponds contaminated with animal urine.

Leptospirosis is a serious emerging waterborne fatal bacterial zoonosis. Large outbreaks occur worldwide. A significant morbidity occurs in tropical areas following flooding and excess rainfall [5]. Hence, large outbreaks of leptospirosis are not restricted only to tropical regions [3]. In China, 50 human casualties were reported in 1500 patients infected with leptospirosis during a 10-month follow-up study [6]. Leptospirosis occurs everywhere carrier rats could be found. Dogs, pigs, horses, cattle, rats, mice, mongoose, and sea mammals could be infected [7]. The symptoms of leptospirosis, also known as Weil's disease, usually manifest 7-14 days after being infected and are extremely broad. It is mostly misdiagnosed because of its nonspecific symptoms, including fever, headache, chills, sweating, muscle pain, painful eyes, jaundice, and vomiting.

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Many organs are involved resulting in nephritis, hepatitis, pneumonitis [8,9], meningitis [10], pancreatitis [11], and erythema nodosum [12], and leptospirosis could result in death if not treated promptly [13].

As leptospirosis was recorded in the Middle East, in particular Turkey and Iran [14,15], a limited number of studies have been performed in Iraq to diagnose the disease in farm animals mainly in Baghdad, Mosul, and Nasiriyah Provinces [16-21].

Alternatively, the role of rats in dispersing leptospirosis was never investigated in Iraq. Because of the seriousness of the disease and the scarce data regarding this disease in Iraq, this study determines the incidence of leptospirosis in rats and its renal histopathological profile.

Materials and Methods

Ethical approval

All tests and procedures were approved under no. 1569 on January 13, 2015, by the Animal Care and Use Committee of the Faculty of Veterinary Medicine, University of Baghdad.

Study period and location

This study was conducted at the Unit of Zoonotic Diseases, College of Veterinary Medicine, University of Baghdad from a period extended from May 2015 till the end of August 2016.

Traps

Small (28 cm×10 cm×10 cm) and large (35 cm×15 cm×15 cm) iron cages were used in this study for capturing rats. Traps, baited with cheese and cucumber, were set in the evening in different sites [22]. Each live trap with captured animal was tagged locally and collected early in the morning. Then, the animals were brought to the laboratory and kept in a big transparent polythene cage that helped to observe their movements for 24 h. Thereafter, the rats were caught from tail, and 0.1 mL of anesthesia (9:1, ketamine + xylazine per 100 g rat body weight) was intraperitoneally injected as described by Struck *et al.* [23].

Animals

Two hundred and eleven black stray rats (*Rattus rattus*) were caught from various areas, including old buildings, markets, and garbage bins in the Baghdad Province. The rats were classified into three groups (juvenile, sub-adult, and adult groups) according to body weight (<100 g, 100-150 g, and >150 g), and the position of the testes (abdominal or scrotal), and status of the vagina (unopened, opened, or scars), as described by Schlafer and Foster [24].

Media, chemicals, and equipment

All media, chemicals, and equipment used in this study were purchased from certified companies (Sigma-Aldrich and Promega, USA).

Dissection of captured rats

After euthanatizing, the outer orifices and median dissecting line of each rat were disinfected with 70%

alcohol. Deep pharyngeal swabs in transport media were taken for bacteriological culture, and then, the rats were dissected individually. The abdominal cavity was opened using a sterile disposable blade under a sterile hood.

Specimens collected

Feces pellets from the rectum and urine from the urinary bladder using a sterile syringe were collected for bacteriological and parasitological investigations [25]. The kidney (0.5 g) was collected under a sterile condition in the hood, placed in a sterile Petri dish, and kept in the deep freeze at -20°C for homogenization. Approximately 0.5 cm³ of the kidney was placed in 10% formalin buffer phosphate overnight. The following day, the solution was discarded and replaced with fresh formalin buffer saline for prompt fixation and processed for routine histopathological investigation. Slides were stained using hematoxylin and eosin (H&E) and Warthin–Starry (WS) stains [26,27].

Blood sampling

After anesthetization, the rats were tested for corneal reflex reaction and toe pinch. The heart was approached easily by laying the rats on their right lateral side. Cardiac puncture was performed using a 5-mL syringe, and 3-5 mL of blood was collected.

Thin blood smears

Immediately, thin blood smears were prepared, and the rest of the collected blood was drained into a sterile plain tube and kept in a fridge at 2-4°C overnight to allow the samples to clot. The thin blood smears were fixed with 100% methanol and let dry completely before staining. The thin blood smears were stained using the WS stain for detecting *Leptospira* [27].

Serum

Clotted blood kept in a fridge overnight was centrifuged at approximately 3000 rpm for 15 min. Serum was removed and transported to an Eppendorf tube and stored at -20°C until analysis.

Rat *Leptospira* immunoglobulin G (IgG) enzyme-linked immunosorbent assay (ELISA) kit

An assay was performed for detecting serum IgG according to the manufacturer's instructions.

Kidney enzymes

Serum blood urea nitrogen (BUN) and creatinine concentrations were estimated, according to Gounden *et al.* [28].

Kidney homogenate

Homogenates were prepared according to Karim [29].

Statistical analysis

The results were expressed as mean ± standard deviation and analyzed using the Chi-square test and one-way analysis of variance. All statistical analyses were performed using Statistical Package for the

Social Sciences version 22 (IBM Corp., Armonk, NY, USA).

Results

Captured rats

Of 211 captured rats, 82 (38.86%) showed negative bacteriological culture and negative parasitological yield. According to Schlafer and Foster [24], the age classes of the rats involved in this study were distributed (Table-1).

ELISA

Plotted standard curve (Figure-1) shows that optic density above 2.0 yielded zero IgG concentrations. All rats were serum IgG-positive (Table-2) with a wide range shown by a high standard deviation (96.172 ± 46.475). Alternatively, kidney tissues showed that 90.24% of the rats had IgG against leptospiral antigens, and the rest (9.76%) were IgG-negative.

Effect of age

All age groups showed that 100% of the rats were serum IgG-positive. Alternatively, kidney homogenates showed that the rats in the juvenile group had higher IgG concentrations than the other age groups (Table-3).

Gender susceptibility

Although significant variations in IgG levels were observed between genders within the same age group, no significant differences in the overall results were observed (Table-4).

Table-1: Classification of captured rats according to age classes.

Age class	No.	Male	Female
Juvenile	44 ^b (53.66%)	19	25
Sub-adult	23 ^a (28.05%)	13	10
Adults	15 ^a (18.29%)	11	04
Total	82	43	39

Different superscripts refer to significant differences ($p < 0.05$) between age groups

Table-2: Concentration (mg/mL) of immunoglobulin G in serum and kidney homogenate of captured rats.

Parameters	Kidney homogenate		Serum	
	Negative	Positive	Negative	Positive
	(n=8)	(n=74)	(n=0)	(n=82)
OD	2.086 ± 0.014	1.659 ± 0.023	Nil	1.243 ± 0.017
Conc.	0	95.172 ± 50.674	Nil	96.172 ± 46.475
%	9.76	90.24	0	100

Table-3: Occurrences of immunoglobulin G in serum and kidney homogenate of captured rats according to age classes.

Age class	n	Kidney homogenate		Serum	
		Positive n (%)	Negative n (%)	Positive n (%)	Negative
Juvenile	44	42 (95.45) ^a	2 (4.55)	44 (100) ^a	Nil
Sub-adult	23	21 (91.30) ^a	2 (8.70)	23 (100) ^a	Nil
Adults	15	11 (73.33) ^b	4 (26.67)	15 (100) ^a	Nil
Total	82	74 (90.24)	8 (9.76)	82 (100)	0

Different superscripts refer to significant differences ($p < 0.05$) between age groups

Kidney function test

Serum concentrations of BUN and creatinine (Table-5) showed significant increments ($p < 0.05$) in the sub-adult and adult IgG-positive groups compared with the IgG-negative groups. No significant alterations were observed in the juvenile group. The alterations in these parameters increased two-fold in the sub-adult group, and BUN peaked as high as threefold in the adult group, whereas the increment for creatinine was almost eightfold.

Detection of leptospires in blood smears and kidney

Using the WS and H&E staining, leptospires in blood smears and renal tissues were not detected in the juvenile group (Table-6). However, 13 positive blood smears were infected with leptospires (Figure-2), whereas no bacteria could be detected in the kidneys in the sub-adult group. In the contrary, only eight kidneys stained using the WS stain (Figure-3) and one blood smear showed the presence of leptospires in the adult group.

Histopathology of the kidney

No specific gross lesion was evident. Microscopical findings of the renal cortex and medulla in the sub-adult

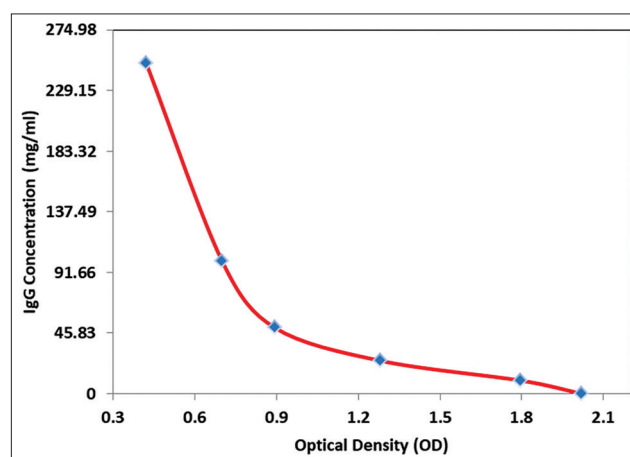


Figure-1: A standard curve showed the optic density versus immunoglobulin G concentrations.

IgG-positive group showed hemorrhage (Figure-4), glomerular deterioration (Figure-5), tubular cell degeneration and necrosis (Figures-6 and 7) with cast formation (Figure-8), periarterial edema, and focal hemorrhage (Figure-9) with congestion of peritubular arteries (Figure-10). Microscopical findings of the renal cortex in the adult IgG-positive group revealed deterioration similar to that in the sub-adult group (Figures-11-13). No leptospires were observed using H&E staining. However, bacteria were observed inside the renal tubules using the WS staining method (Figure-3).

Discussion

Captured rats

Health status

Among the 211 captured rats, 82 (38.86%) rats showing negative bacteriological culture and negative parasitological yield were included in this study. This action was taken to exclude any pathological effects on the various parameters (kidney function tests and histopathology) planned to be investigated. Wild and laboratory rats are usually prone to be infected with different diseases [30-32]. Although complete

coverage of the health status is not fulfilled, 38.86% of the captured rats were apparently healthy. This ratio conforms to the study by Clement *et al.* [33] who reported higher rates of apparently healthy rats based on clinical examination only.

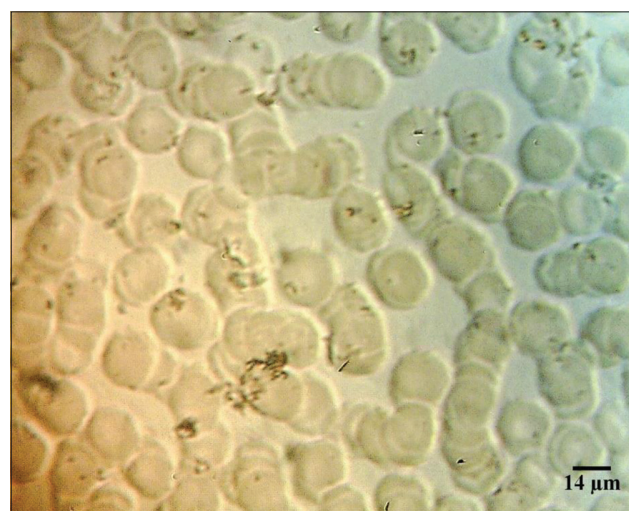


Figure-2: Blood smear of sub-adult immunoglobulin G positive rats. Note the presence of *Leptospira* (WS stain).

Table-4: Occurrences of immunoglobulin G in serum and kidney homogenate of captured rats according to gender.

Age class (N)	Immunoglobulin G	Kidney homogenate			Serum		
		n	Male	Female	n	Male	Female
Juvenile (44)	Positive	42	19 (43.2%) ^{aB}	23 (52.3%) ^{bA}	44	19 (43.2%) ^{aA}	25 (56.8%) ^{bA}
	Negative	02	0	2 (4.5%)	0	0	0
Sub-adult (23)	Positive	21	12 (52.2%) ^{aA}	9 (39.2%) ^{bB}	23	13 (56.5%) ^{aB}	10 (43.5%) ^{bB}
	Negative	2	1 (4.3%)	1 (4.3%)	0	0	0
Adults (15)	Positive	11	08 (53.3%) ^{aA}	3 (20.0%) ^{bC}	15	11 (73.33%) ^{aC}	4 (26.66%) ^{bC}
	Negative	4	3 (20.0%)	1 (6.7%)	0	0	0
Total (82)	Positive	74	39 (47.6%) ^a	35 (42.6%) ^a	82	43 (52.4%) ^a	39 (47.6%) ^a
	Negative	8	4 (4.9%) ^a	4 (4.9%) ^a	0	0	0

Different small superscripts refer to significant differences ($p < 0.05$) within same age group.

Different large superscripts refer to significant differences ($p < 0.05$) between age groups

Table-5: Concentrations of BUN and Creatinine in serum of captured rats.

Age class (N)	Immunoglobulin G	BUN (mg/dL)	Creatinine (mg/dL)
Juvenile (44)	Positive	8.43±1.09 ^a	0.91±0.16 ^a
	Negative	11.37±2.32 ^a	1.12±0.27 ^a
Sub-adult (23)	Positive	21.56±2.87 ^b	2.89±0.31 ^b
	Negative	11.37±2.32 ^a	1.12±0.27 ^a
Adults (15)	Positive	36.25±5.13 ^c	8.26±1.69 ^c
	Negative	11.37±2.32 ^a	1.12±0.27 ^a

Different superscripts refer to significant differences ($p < 0.05$) between age groups. BUN=Blood urea nitrogen

Table-6: Presence of leptospires in kidney and blood smear.

Age group	Immunoglobulin G	n	Blood smear	Kidney	
				Hematoxylin and eosin	WS
Juvenile (44)	Positive	42	-	-	-
	Negative	02	-	-	-
Sub-adult (23)	Positive	21	13	-	-
	Negative	02	-	-	-
Adults (15)	Positive	11	1	-	8
	Negative	04	-	-	-
Total (82)	Positive	74	15	-	-
	Negative	8	-	-	-

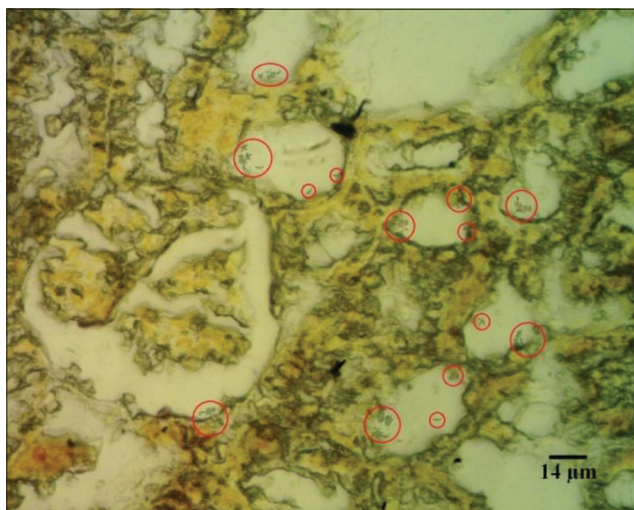


Figure-3: Microscopic findings of kidney of sub-adult immunoglobulin G positive rats. Note the presence of *Leptospira* inside glomeruli (red circles) (WS stain).

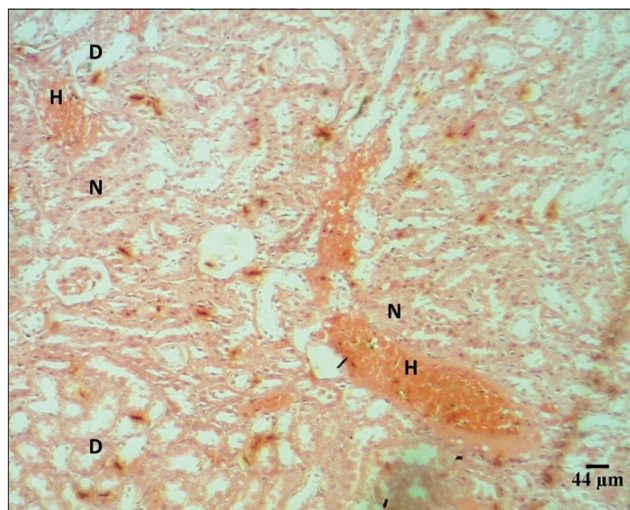


Figure-6: Microscopic findings of kidney cortex of sub-adult immunoglobulin G positive rats. Note the hemorrhage (H), tubular cells degeneration (D), and necrosis (N) (hematoxylin and eosin, stain).

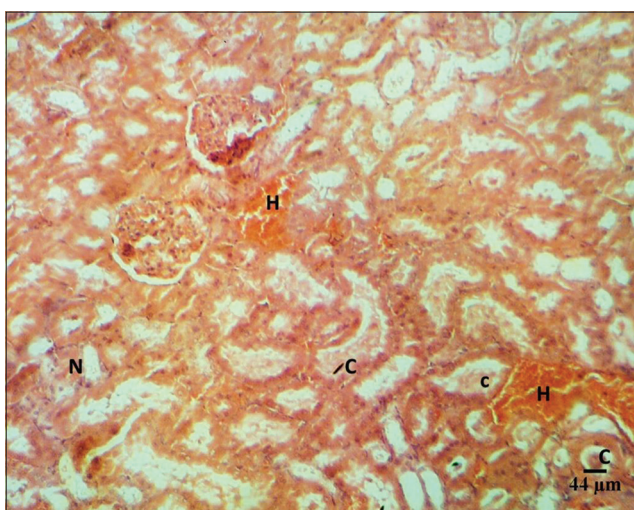


Figure-4: Microscopic findings of kidney cortex of adult immunoglobulin G positive rats. Note the Hemorrhage (H) and congestion (C) (hematoxylin and eosin, stain).

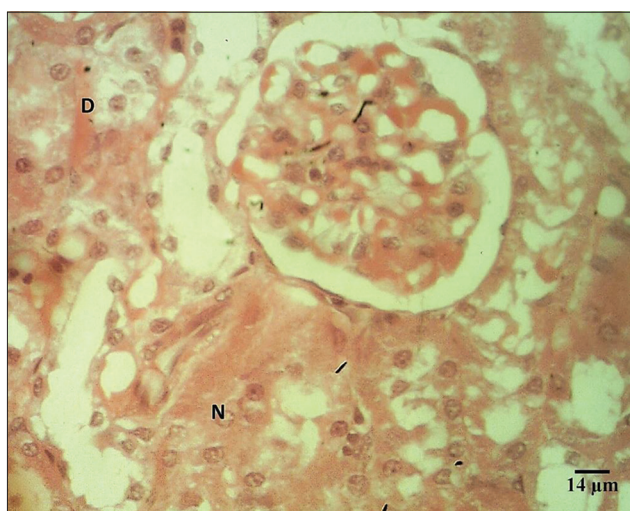


Figure-7: Microscopic findings of kidney cortex of sub-adult immunoglobulin G positive rats. Note the tubular cells degeneration (D) and necrosis (N) with normal glomerulus appearance (hematoxylin and eosin, stain).

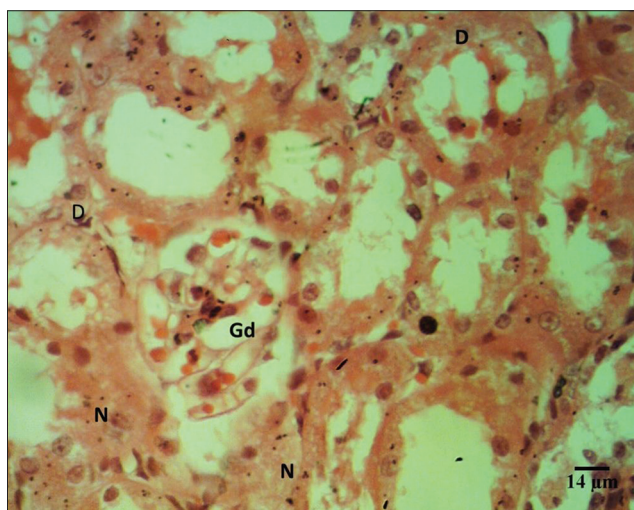


Figure-5: Microscopic findings of kidney -cortex of sub-adult immunoglobulin G positive rats. Note the tubular cells degeneration (D) and necrosis (N) with glomerular deterioration (hematoxylin and eosin, stain).

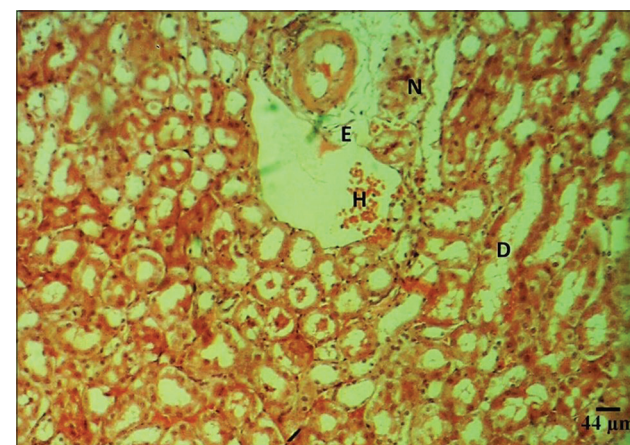


Figure-8: Microscopic findings of kidney medulla of sub-adult immunoglobulin G positive rats. Note the tubular cells degeneration (D) and necrosis (N) with cast formation (hematoxylin and eosin, stain).

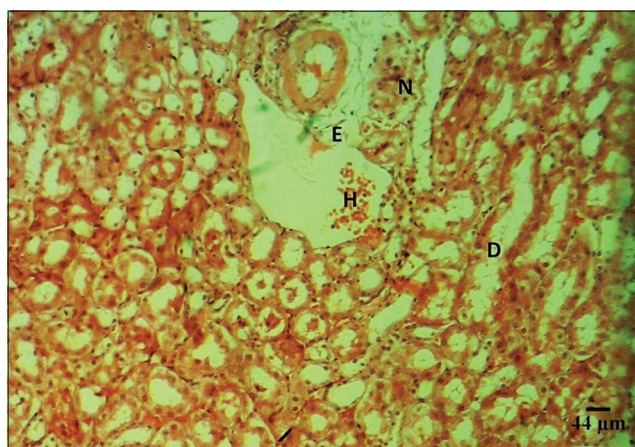


Figure-9: Microscopical findings of kidney of sub-adult immunoglobulin G positive rats. Note the tubular cells degeneration (D) and necrosis (N) with periarterial edema (E) and hemorrhage (H) (hematoxylin and eosin, stain).

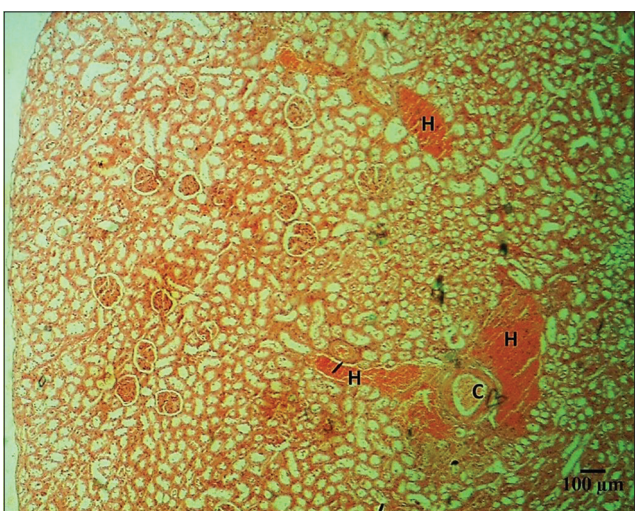


Figure-10: Microscopical findings of kidney of sub-adult immunoglobulin G positive rats. Note the focal hemorrhage (H) with congestion of peritubular arteries (C) (hematoxylin and eosin, stain).

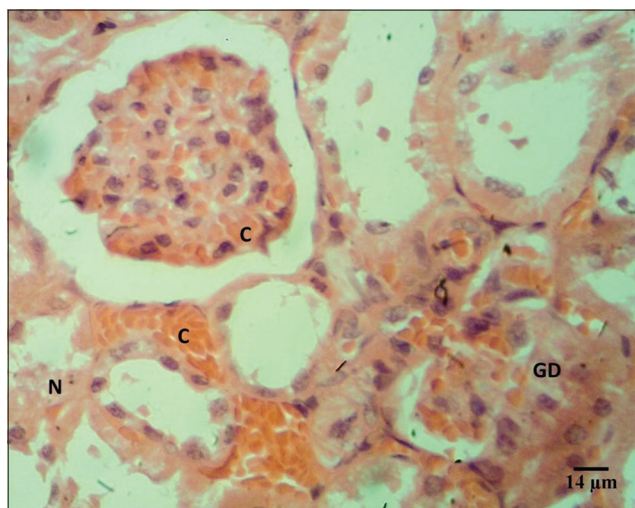


Figure-11: Microscopical findings of kidney-cortex of adult immunoglobulin G positive rats. Note the congestion of peritubular arteries and glomerular tuft congestion (C), tubular necrosis (N), and glomerular deterioration (GD) (hematoxylin and eosin, stain).

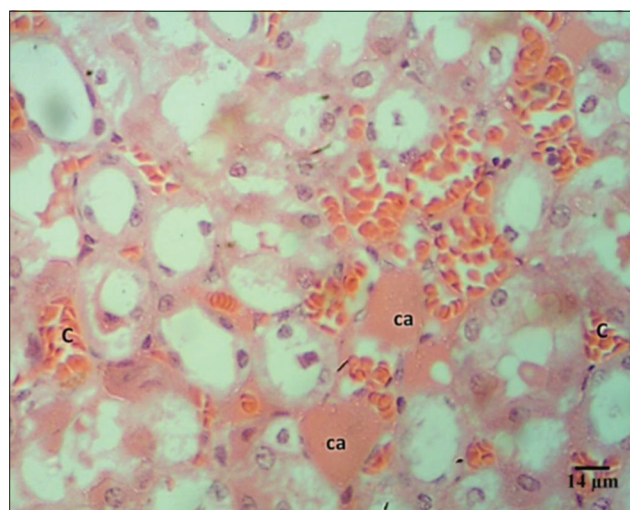


Figure-12: Microscopical findings of kidney of adult immunoglobulin G positive rats. Note the peritubular arterial congestion (C) and cast formation (ca) (hematoxylin and eosin, stain).

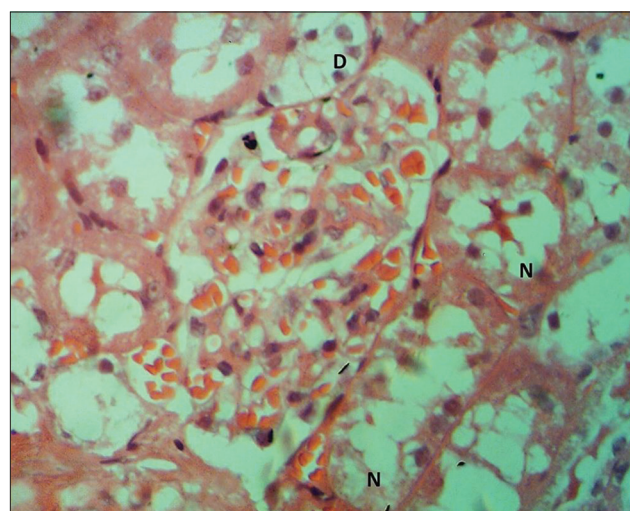


Figure-13: Microscopical findings of kidney-cortex of adult immunoglobulin G positive rats. Note the tubular degeneration (D) and necrosis (N) with glomerular deterioration (hematoxylin and eosin, stain).

Age of captured rats

The use of age groups in this study conforms to the study by Choo *et al.* [34]. Despite that the number of males and females was equal (Table-1), considering the distribution of age groups, the juvenile group composed of significantly the largest number of rats. This might be explained by the fact that juvenile rats are not experts in collecting their food and look at the surroundings differently from the adult rats [34].

ELISA

Serum IgG was positive in all captured rats. This either refers to a current infection, chronic infection, or acquired IgG from the dam through milk [35,36]. Immunity following leptospiral infection is humorally mediated against lipopolysaccharides and antigenically restricted to related serovars [37]. De Oliveira *et al.* [35] have argued that *Leptospira* spp. was

detected in the mammary glands and milk of naturally infected rats, which coincided with the presence of leptospires in the kidneys of all rats in this study, indicating chronic carriage. Records in Table-2 indicate that 90.24% of the rats were IgG-positive in kidney homogenates. Although this rate is too high comparable to the findings of Heuser *et al.* [38], who detected *Leptospira* DNA in 14.3% of rats using polymerase chain reaction (PCR), this wide spectrum of variation may be attributed to the detection of the leptospire antigens in a later study, whereas this study was confined with the detection of antibodies.

Passive immunity, provided by the dam through the placenta and milk mainly containing IgG and IgA antibodies, offers protection against many bacterial and viral diseases [39]. The neonatal mammalian gut could absorb IgG for hours to days after birth. Failure of passive transfer of IgG can be fatal due to the lowered amount of maternal IgG in the blood to resist common diseases. Maternal IgG is correlated with the amount of suckled maternal antibody by the neonate referring to that higher titers persist for a longer time. The high concentration of IgG in the serum and kidney homogenates in juvenile rats is explained in this view (Table-4). Maternal antibodies usually persist for 3-6 months in domestic animals, only 4-7 days in chickens, and 6-12 months in humans [40,41]. Despite the prediction, that maternal immunoglobulins are passed through milk to the neonates, the seroprevalence of leptospires was higher than the detection of antigens. This was confirmed by a study performed on rats trapped in Barbados showing higher seropositivity prevalence rates of 34% in *R. norvegicus* and 30% in *R. rattus*, but lower identification rates using dark-field microscopy at 27% and 15%, respectively [42]. These records conform to the findings in this study, although leptospire detection was not performed. Dreyfus *et al.* [43] have shown that the high seroprevalence (35%) suggests an ongoing exposure. This suggestion potentiates the risk of a current infection among the rats studied.

The rate of *Leptospira* carriage in small mammals trapped in Eastern Croatia was high (29.9%), which corresponds to the high incidence of human and domestic animal leptospirosis [44]. In another study, Hagan *et al.* [45] have revealed that *Leptospira* spp. was the most common pathogen in rats trapped in Brazil, which peaked at 90% as a presumptive infection based on serology. The prevalence of *Leptospira* spp. increased significantly in rats, which was comparable to that of Seoul virus and *Bartonella* spp. They have suggested differences in the transmission dynamics of these different pathogens, indicating the high prevalence of leptospiral carriage by rats and a high degree of risk to human health [45]. In humans, serological diagnosis using the microscopic agglutination test (MAT), IgM and IgG ELISA confirmed leptospirosis in children with acute febrile illnesses in Tanzania. The prevalence recorded was 13-41% in those who

reside with rats with the ugliest danger risk reported; more than 95% of patients specifically reacted to agglutination using the MAT [46-48].

The high occurrence rates of antibodies in the serum and kidney homogenates (Table-4) could explain that rats are sharing the same contaminated environment leading to the preponderance of common asymptomatic infections in humans in endemic areas [49]. ELISA tests and PCR may play a key role in the early detection and treatment of human leptospirosis in developing countries [50]. Blanco *et al.* [51] have correctly identified infecting serogroups using MAT matching with the identification of isolates, which fit the epidemiological surveys. Lau *et al.* [50] have reported that IgM ELISA, real-time PCR, cPCR, and IgG ELISA had the greatest diagnostic accuracy. Due to the species of the rats used in this study, caring for the acute diagnosis of the disease was ignored. Alternatively, this study confirmed the usefulness of IgG ELISA for diagnosing either present or previous infection.

Comparing our serology findings with those of other studies on small ruminants, the seroprevalence in rats was 100% and 90.24% in the serum and kidney homogenates, respectively (Table-2). Sheep and goats residing in tropical St. Croix, US Virgin Islands showed seroprevalence rates of 32% and 26%, respectively [52], which are comparable to 6.1% (sheep) and 2.1% (goat) in Italy [53]. The wide difference in seroprevalence may be attributed directly to the tools used for diagnosing [54]. The seroprevalence of leptospirosis in ovine livestock detected using the MAT in Tunisia was 25%, whereas it reached 70% at the herd level. This evidence confirmed the chronic and active circulation of *Leptospira* in ovine, which may act as reservoirs or incidental hosts in which the productivity of small ruminants is significantly reduced [55-57]. The high endemicity (5.6%) in Thailand, up to 25% in Colombia, was recorded for leptospirosis in rats trapped in urban and rural areas using the MAT [58,59]. These low prevalence rates detected using the MAT compared with our findings regarding ELISA IgG2 (Table-2) evoke the necessity of using more than one tool in diagnosing leptospirosis. The sensitivity of ELISA is 72% (comparable to that of the MAT) during acute illness, which is lowered to <25% during the critical 1st week of illness when antibiotic therapy is most effective [60-62]. The high seroprevalence rate reported in this study predicts that rats play a key role in dispersing *Leptospira* in its surroundings.

Young animals affected more than other

The highest occurrence of leptospiral IgG was recorded in the juvenile group (Table-3). Whether serum IgG in neonates acquired from the dam or from a valid infection, many researchers refer to younger age to be the most susceptible. This was confirmed in rats [45], lambs [56], dogs [63], piglets [64], and children [47,48]. In addition to the milk route, leptospirosis

could be transmitted to neonates through semen [65], causing IgG induction, although some of these antigens might be neutralized by maternal IgG [64]. The former facts potentiate our findings regarding the elevated IgG concentration in juvenile rats.

In humans, all ages are susceptible, and the highest incidence of leptospirosis occurred in individuals aged above 55 and 65 years in both genders [47]. Ciceroni *et al.* [53] have found that all age groups (15-64 years) were prone to infection, but infection was more common in the working-age population [53]. Similar findings were reported by Holk *et al.* [66] who recorded 93% of the patients aged 18-64 years. Infection was reported in the lower age individuals (15-34 years) who reside close to an open sewer [47,67] or individuals aged between 19 and 29 years performing piggery-farming activities [68]. The mortality rate is higher in the elderly than that in young adults who are more prone to subclinical leptospirosis [69].

Gender susceptibility

The presence of IgG in the serum or kidney homogenates varies between genders (Table-4). No significant difference in the total IgG value in kidney homogenates and the serum between males (47.6% and 52.4%, respectively) and females (42.6% and 47.6%, respectively). This might be explained by the fact that both genders are exposed to the same pathogenic serotype and same environmental conditions. This coincided with the results of a study that has reported that gender-related differences were not associated with exposure risk, infecting serovars, or health-seeking behavior [70]. Many sero-epidemiologic studies have reported that males are 3 times more likely to be infected with leptospirosis with a higher severity than females, whereas death from leptospirosis was nearly twice as common among females [70,71]. Thus, leptospirosis predominance in males reflects gender variations in the incidence of severity of the disease, rather than difference in infection rates.

As for many other diseases, including tuberculosis, listeriosis, and amebiasis [72-75], gender may influence the severity and outcome of clinical leptospirosis [76]. Males have increased susceptibility to not only leptospirosis but also many other infections and the development of diseases [72-74]. This was attributed in part to the effect of estrogen [73]. Conventionally, the altitude in male incidence is explained by higher exposure to risk factors associated with occupations typically confined to males, such as butchering, livestock farming, rice farming, and fishing. Alternatively, a major cause of leptospirosis emerged as travel-related in developed countries [77]. In the Netherlands, Goris *et al.* [78] have reported that 91.1% of male patients are infected with leptospirosis compared with 8.9% for female patients. A higher incidence rate of leptospirosis (Male/Females=5.4) in males than that in females was recorded in the Pacific [78]. In addition, males showed severe leptospirosis infection with

6.9% died compared with 2.7% in females [77]. The incidence of leptospirosis cases was 74%–90% among men [66,79], and the mortality rate among males was higher than that among females [53,67]. Whether it only reflects higher exposure of male gender due to their responsibilities in agricultural and farming activities or sex-specific susceptibility factors is not yet confirmed.

Kidney function tests

Renal function is clinically estimated using the glomerular filtration rate. Levels of BUN and serum creatinine are considered important indicators of kidney health [80]. Serum creatinine is the most commonly used tool for the indirect detection of marked damage to functioning nephrons and indicates late-stage renal dysfunction [81]. The removal of creatinine from the blood is chiefly performed by glomeruli and proximal tubules in the kidneys. Deficient renal filtration of creatinine raises its blood levels. Therefore, serum and urine creatinine levels may be used as indicators of creatinine clearance. Myositis, a common clinical feature in leptospirosis usually fading by the 2nd week, is linked to high creatine phosphokinase (CPK) levels [71]. The increased creatinine levels in both the sub-adult and adult groups in this study were confirmed by many studies linking leptospirosis to extremely high CPK levels [80-82]. CPK determination is a simple test that may provide diagnostic information in a jaundiced patient, particularly when characteristic manifestations of leptospirosis are absent, suggesting a diagnosis of leptospirosis [82]. Moreover, Knopfler *et al.* [81] have supported our results. They have argued that creatinine and BUN are markedly elevated in dogs, equine, and Wistar rats with acute leptospirosis [82,83]. In humans, renal, and pulmonary involvement is commonly present in acute leptospirosis. In renal involvement, creatinine exceeding 3.0 mg/dL is a strong predictor of death [70]. Our study showed that creatinine reached this value in the sub-adult group and approximately 3 times in the adult group (Table-5). This finding revealed the degree of damage inside the kidneys and might explain the shorter life span of the wild rats than the laboratory rats due to renal failure. Nonetheless, the collaboration of a marked increase in creatinine level with damage to renal tubules may present a reliable understanding of renal failure that predicts a lethal outcome in severe leptospirosis [70]. BUN concentration is a less specific marker of kidney function than serum creatinine [83]. Our findings (Table-5) revealed 2-3 times increment in BUN in the sub-adult and adult IgG-positive groups comparable to those in seronegative rats. Studies have shown that if urea and creatinine are increased, a minimum of 75% of the nephrons must have lost their function [83].

Histopathology

It was suggested that in acute leptospirosis, the pathogenesis of the pathological features is related

to the presence of organisms in the tissues [84]. This was evident in our observations. The presence of leptospires in the blood smears (Figure-2) indicates the acute phase of leptospirosis in the sub-adult group (Table-6 and Figure-3). The rest of the sub-adult IgG-positive rats were suspected to have a continuation of maternal immunity or a valid infection. This cannot be confirmed unless a PCR is performed [54,84]. In acute leptospirosis, the main pathogenesis was probably due to the direct toxic damage of leptospires [71]. Systemic lesions in leptospirosis are caused by direct damage to the parenchymal cellular membranes, causing functional disorder, and necrosis. This deterioration may predispose patients to hemophagocytosis and disseminated intravascular coagulation, which are dominant in Gram-negative bacterial diseases but rarely present in human leptospirosis [82,85].

Using H&E staining, the kidneys exhibited histopathological changes manifested by interstitial nephritis that predominantly consisted of lymphocytes (Figures-4-13). Tubulointerstitial nephritis is a main renal manifestation caused by pathogenic *Leptospira* that mostly accumulates in the proximal tubules, by a toxic component in the outer membrane (e.g., 32-kD lipoprotein, which is absent in the nonpathogenic *Leptospira*), cause macrophage accumulation and disrupted the basolateral location of Na-K-ATPase in pronephric ducts resulted in substantial impairment in the proximal tubular defect without affecting distal nephrons [86]. Mortality due to acute renal failure is approximately 15-20% in association with the presence of higher levels of creatinine [87].

Detecting the presence of leptospires in urine or in blood or tissues is difficult [88]. Many authors have detected leptospires in various body fluids of cattle, sheep, and goat using dark-field microscopy and confirmed by PCR, without bacterial isolation [62]. Our tools for detecting leptospires in the blood smears and kidneys were using silver impregnation (WS) and H&E stains. The outcome of these staining methods to identify the bacteria in the same rat varied, but only one rat from the adult group revealed a positive blood smear coincided with the presence of leptospires in the kidney (Table-6).

The occurrence of antibodies against many serovars without leptospiral isolation cannot establish a diagnosis of the disease [88], even during the early acute phase of the disease, where IgM ELISA can give a presumptive diagnosis of leptospirosis [89]. Unlike ELISA, PCR can accurately confirm the disease. Rojas *et al.* [90] have confirmed leptospires that colonize the renal tubules in the urine of 7.05% of chronically infected dogs using PCR. Agudelo-Flórez *et al.* [59] have reported that even in WS staining-positive kidneys, negative bacterial isolation was obtained from H&E staining and culture or even from PCR-LipL32 on *R. norvegicus* naturally infected from an urban area in Colombia. Several factors may prevent the growth of *Leptospira* in the specimens.

For example, the acidity of urine and the presence of specific antibodies and other bacterial contamination may directly interfere with the growth of leptospires and their isolation [91].

Renal involvement is common in leptospirosis, characterized by tubulointerstitial nephritis (Figure-6), resulting in tubular dysfunction. Yang [91] has reported that leptospiral outer membrane proteins (OMPs) may elicit tubular injury and inflammation through the Toll-like receptor-dependent pathway followed by the activation of nuclear transcription factor-kappa B and mitogen-activated protein kinases motivated by differential induction of chemokines and cytokines relevant to tubular inflammation. *Leptospiral* OMP may also induce the activation of the transforming growth factor-beta/Smad-associated fibrosis pathway leading to the accumulation of extracellular matrix [91]. Fanton d'Andon *et al.* [92] have argued that renal colonization of *Leptospira* could induce mild renal fibrosis through TLR- and NLR-independent pathways, and the activation of iNOS plays a role in the initiation of renal fibrosis. Fibrosis results in structural and functional renal alterations characterized by excessive accumulation of scarring tissues, which may lead to organ dysfunction [6]. In addition, Lau *et al.* [50] have demonstrated leptospires in the renal tubules of seropositive cows (12.5%) using WS staining, which conforms to the studies by Yener and Keles [93] and Saglam *et al.* [94]. Interstitial nephritis, tubular atrophy, and glomerular lesions of minimal intensity described in our results conform to the findings of many authors [50,95].

Conclusion

IgG-positive carrier rats refer to previously exposed or infected rats. The risk of transmitting the disease to humans and animals through urine is highly predicted and possible mitigation of zoonotic transmission.

Authors' Contributions

AJK contributed to the design of the study, writing, analyzed data, and revision. ZMA performed the experiment, analyzed data, and wrote the draft. Both authors read and approved the final manuscript.

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Competing Interests

The authors declare that they have no competing interests.

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