ISSN: 2456-2912
VET 2024; SP-9(1): 580-585

www.veterinarypaper.com
Received: 02-10-2023
Accepted: 09-11-2023

Shivaraj Murag
Associate Professor, SRDDL, IAH&VB, KVAFSU, Hebbal, Bangalore, Karnataka, India

Manjunath Reddy
Assistant Professor, (OPG), WRDDL, IAH&VB, KVAFSU, Bangalore, Karnataka, India

Sanjeev Kumar M
Assistant Professor, SRDDL, IAH&VB, KVAFSU, Hebbal, Bangalore, Karnataka, India

Shivashankar BP
Associate Professor, SRDDL, IAH&VB, KVAFSU, Hebbal, Bangalore, Karnataka, India

Sripad K
Professor, SRDDL, IAH&VB, KVAFSU, Hebbal, Bangalore-24, Karnataka, India

Parijatha S Khoday
Research Fellows, IAH&VB, KVAFSU, HEBBAL, Bangalore, Karnataka, India

Manohar Raju MV
Research Fellows, IAH&VB, KVAFSU, HEBBAL, Bangalore, Karnataka, India

Bhavana
Research Fellows, IAH&VB, KVAFSU, HEBBAL, Bangalore, Karnataka, India

Raveendra Hegde
Director, IAH&VB, KVAFSU, Hebbal, Bangalore-24, Karnataka, India

Corresponding Author:
Shivaraj Murag
Associate Professor, SRDDL, IAH&VB, KVAFSU, Hebbal, Bangalore, Karnataka, India

Diagnosis of leptospirosis in hyena

Shivaraj Murag, Manjunath Reddy, Sanjeev Kumar M, Shivashankar BP, Sripad K, Parijatha S Khoday, Manohar Raju MV, Bhavana and Raveendra Hegde

Abstract
Leptospirosis, a global disease of animals due to its widespread and potentially fatal zoonosis. Leptospirosis is endemic in most tropical regions causing outbreaks after heavy rainfall and floods affecting domestic, and wild animals as well as humans. Although some studies describe the occurrence of leptospirosis in captive animals. Infected domestic or wild animals act as reservoirs of leptospires, infections resulting from direct or indirect contact with an infected animal by shedding pathogenic leptospires in their urine. Nevertheless, the real role of wildlife animals as a source of infection to livestock and humans, as well as the most important reservoirs and leptospiral strains remains unclear. In places such as zoos and parks, most of the animals should be captivated by restrictions or it can lead to numerous infections and may cause a wild variety of zoonotic diseases. In the current study, differential diagnosis was carried out for the hyena which was found to be dead with hematemesis (blood vomit). The samples were subjected to septicaemic bacterial isolation in Blood agar, semisolid EMJH media and histopathological examination for microscopic changes. EMJH media showed the formation of Dinger's ring after two weeks of incubation and subjected to Polymerase Chain Reaction (PCR), targeting 16S ribosomal G1, G2 (secY gene) and outer membrane *LipL* 32. P-+CR-amplified products were examined in 2% agarose gel. The amplicons from the kidney and heart showed base pairs of 285 for (G1&G2) and 756 for (*LipL32*) respectively indicating that the collected sample is positive for leptospira. On histopathological examination of the kidney showed interstitial nephritis. Thus, based on PCR, Gross Pathology and Histopathological studies it was concluded that the Hyena has died due to Leptospirosis.

Keywords: Hyena, Leptospira, PCR, *LipL*32, histopathology

1. Introduction
Leptospirosis has emerged as an important zoonotic disease globally. It is a bacterial zoonosis, affecting wild animals, domestic as well as humans. It is caused by a spirochete which belongs to Leptospiracea family and genus Leptospira. Leptospira are flexuous and helical microorganisms with one or two hook ends of the size of 0.1 to 0.2 µm by 6 to 20 µm. leptospira colonizes in kidneys of an infected animal (domestic/wild) and these live bacteria are excreted in the urine (Levett, 2001) [5]. The organism has the ability to thrive out of the host for a limited period of time depending upon the warm or cold wet weather conditions. In the Northern hemisphere leptospirosis peaks during late summer to early autumn (August-October). There are more than 200 pathogenic strains of leptospira *viz* serovars and are placed in 23 serogroups on the basis of antigenic relationships. Each of the variants may be adapted to a certain animal, the natural host. Schematically, hosts can be divided into natural and accidental hosts. The host infected with leptospira may show mild or no symptoms after infection and acts as an infectious reservoir for a very long duration and sometimes for the rest of their lives. In certain cases, after infection, accidental hosts might develop severe illness and may also lead to death of the host. In humans, it causes Weil's syndrome, characterized by bleeding, jaundice and failure of kidneys to produce urine (anuria/oliguria) due to impaired function of the liver and kidneys. In case of animals, chronic infection may lead to the failure of kidneys with reproductive problems. Accidental hosts shed leptospires in their urine for a relatively short period. Hence, they do not contribute significantly to the transmission of leptospirosis in terms of maintaining a reservoir. Zoos have a great importance in conserving endangered species, although some studies describe the occurrence of leptospirosis in captivity, many wild animals act as reservoirs of leptospires.

Nevertheless, the real role of wildlife animals as a source of infection to livestock and humans, as well as the most important reservoirs and leptospiral strains remains unclear.

The disease assumes an important epidemiological role because it affects domestic and wild species. In places such as zoos and parks several species must live in restricted areas. This condition can disseminate numerous infectious agents that may cause wild variety of zoonotic diseases (Rahman *et al*., 2020) [8]. In the current study, there was a sudden death of a male hyena with hematemesis (blood vomit), aged about four years at Bannerghatta National Park, Bangalore. On post-mortem examination, it was found that there were pinpoint haemorrhages on lungs and heart. The kidneys were highly congested and the other vital organs showed mild pinpoint haemorrhages. Major morphological changes were noticed in kidneys suggesting that the animal had died due to renal failure (Nephritis) and possible septicaemia. The samples of vital organs like kidneys, liver, heart and lungs were collected and examined for bacterial isolation, identification and histopathological examinations.

2. Materials and Methods

2.1 Materials

BD-Difco's Leptospira Medium Base (EMJH) along with leptospira enrichment was prepared as per the manufacturer's instruction. Blood agar plates were from HiMedia. DNA extraction from the samples was done using QIAamp DNA mini kit – Qiagen (Cat. No: 51306). Oligonucleotides listed in (Table 1) were synthesized and procured from Eurofinn, Bangalore and reconstituted in 1X TE buffer, PCR master mix were from Ampliqon.

2.2 Methods

The samples were subjected to routine septicaemic bacterial isolation in Blood agar, EMJH media for Leptospira with filtration, followed by conventional PCR for leptospirosis targeting two different genes, i.e., G1 and G2 – 16S ribosomal (partial secY gene) and outer membrane *Lip L 32*. The DNA extraction from the samples was done using QIAamp DNA mini kit – Qiagen, following the manufacturer's instructions. The purity of the DNA extracted from clinical materials was estimated using NanoDrop spectrophotometer. The 0.5 µg DNA was used to check the purity by electrophoresis on 0.8 per cent agarose gel and finally, the DNA bands were visualized with a UV transilluminator. The isolated DNA from clinical samples were subjected to PCR, PCR tube containing 12.5 µl of Master Mix, 1.0 µl Forward and Reverse Primers (10 pmole /µl), 4.0 µl of template DNA/ test sample DNA and 6.5 µl of nuclease-free water of final volume 25.0 µl (Table 2). PCR was carried out with *Leptospira interrogans* serovar *sejroe* as a positive control and *Staphylococcus* species DNA as a negative control. PCR amplification conditions targeting G1 and G2 are, the template DNA was initially denatured at 94 °C for 5 min, followed by 32 cycles of 94 °C for 60 s, 55 °C for 60 s, and 72 °C for 120 s, and then 72 °C for 6 min for final extension. To target *Lip*L32, initial denaturation at 95 °C for 5 min, followed by 34 cycles of 95 °C for 60 s, 55 °C for 60 s, and 72 °C for 60 s, and then 72 °C for 10 in for final extension (Table 3).

Table 1: List of primers used for PCR amplification

Primer / Probe	Sequence (5'– 3')	Amplicon size (bp)	Annealing Temp (ºC)	Reference
G1-F	CTG AAT CGC TGT ATA AAA GT	285	55	(Gravekamp *et al*., 1993) [3]
G2-R	GGA AAA CAA ATG GTC GGA AG			
LipL32-F	CATATGGGTCTGCCAAGCCTAAA	756	55	(Meenambigai *et al*., 2011) [6]
LipL32-R	CTCGAGTTACTTAGTCGCGTCAGAA			

Table 2: Reaction components of PCR Mix

Reaction mixture	Volume
Master Mix	12.5 µl
Forward Primer (10 pmole /µl)	1.0 µl
ReversePrimer (10 pmole /µl)	1.0 µl
Template DNA	4.0 µl
Nuclease free water	6.5 µl
Total	25.0 µl

Table 3: The programme followed for PCR amplification

	Primer G1 and G2		Primer LipL32	
	First cycle	Next 32 cycles	First cycle	Next 34 cycles
Denaturation	94 ºC for 5 min	94 ºC for 1 min	95 ºC for 5 min	95 ºC for 1 min
Annealing		55 ºC for 1 min		55 ºC for 1 min
Extension		72 ºC for 2 min		72 ºC for 1 min
		Final extension of 72 ºC for 6 min		Final extension of 72 ºC for 10 min
Number of cycles	33		35	

Subsequently, the samples were subjected to Histopathological examination to understand the microscopic changes in different organs.

3. Results

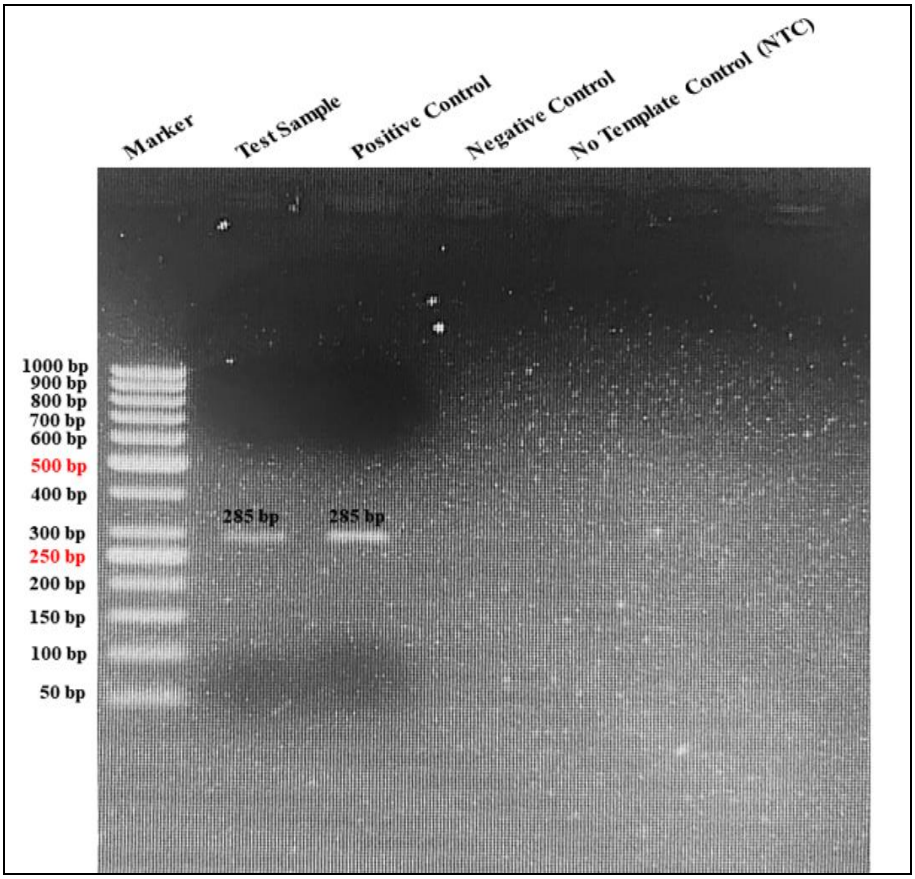

Fig 1: PCR amplification of Leptospiral DNA using primer sets G1/G2. Expected PCR product size of 285 bp obtained using both primer sets for the strains belonging to Leptospira in lane 2 for test sample.

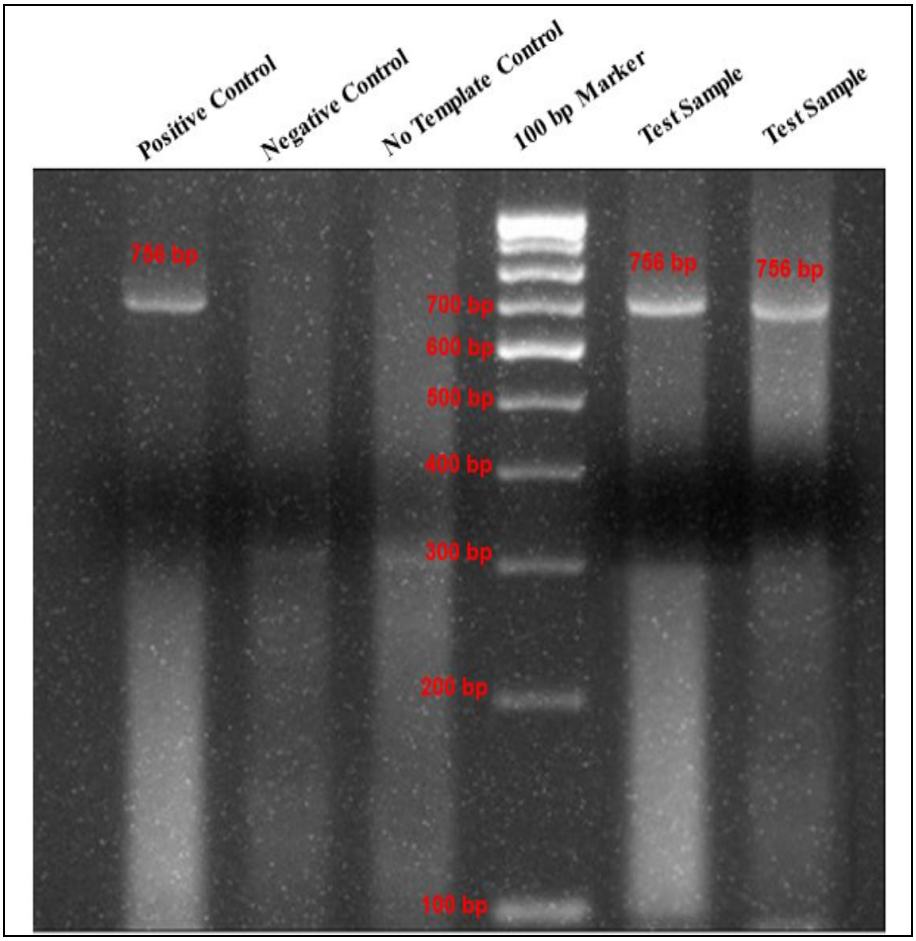

Fig 2: PCR amplified LipL32 gene. Agarose gel of 2.0% showing amplified LipL32 gene from Leptospira. Lane 1: Positive Control (756 bp), Lane 2: Negative Control, Lane 3: No Template Control (NTC), Lane 4: 100 bp Marker, Lane 5 and Lane 6 are test samples (756 bp)

Fig 3: Gross Pathology of Hyena Kidney showing the severe haemorrhages and congestion of the medulla and cortex.

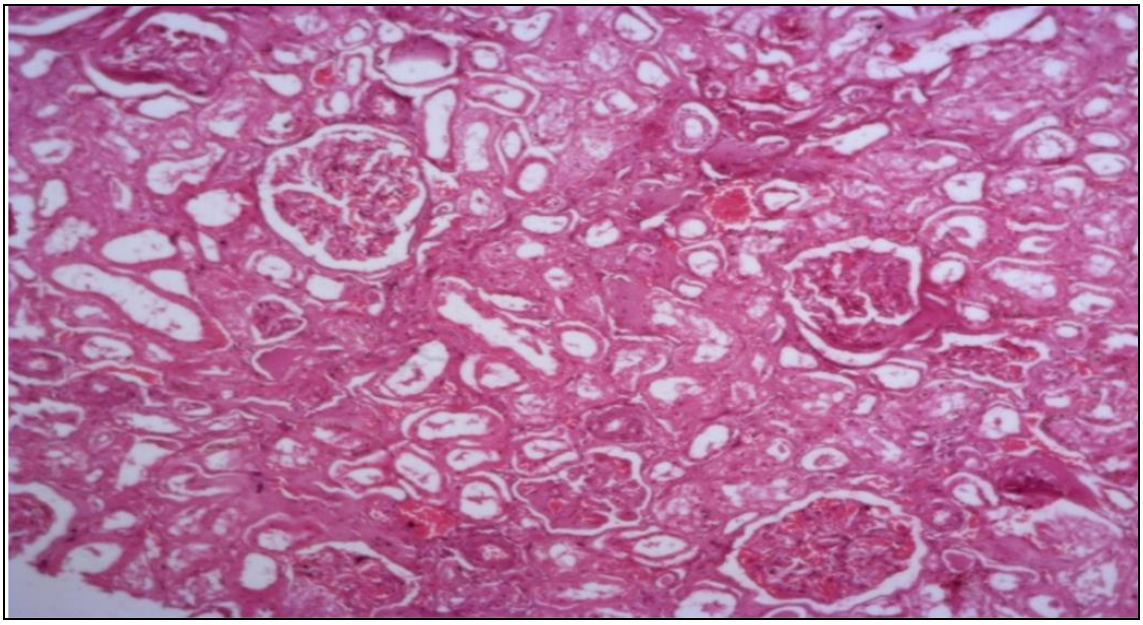

Fig 4: Hyena, Kidney microscopic 10x image, showing the renal nephritis

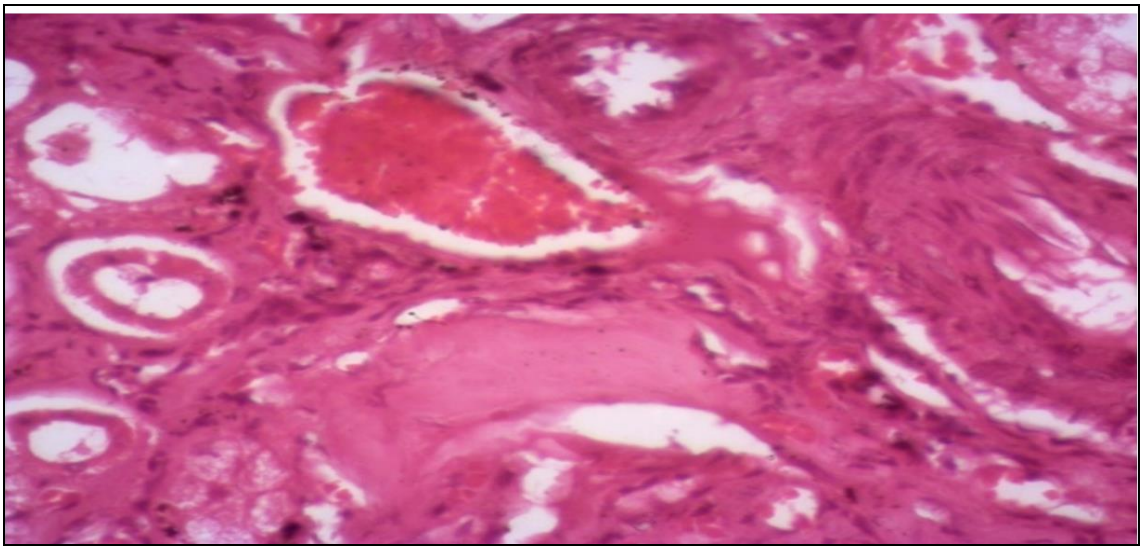

Fig 5: Hyena, Kidney microscopic 40x image, showing the renal fibrosis, renal vascular congestion, tubular edema, and renal tubular degeneration

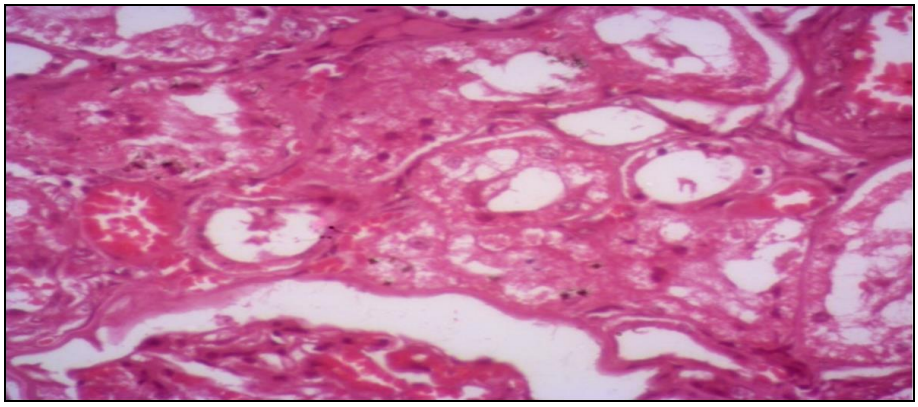

Fig 6: Hyena, Kidney microscopic 40x image, showing the renal fibrosis, interstitial nephritis.

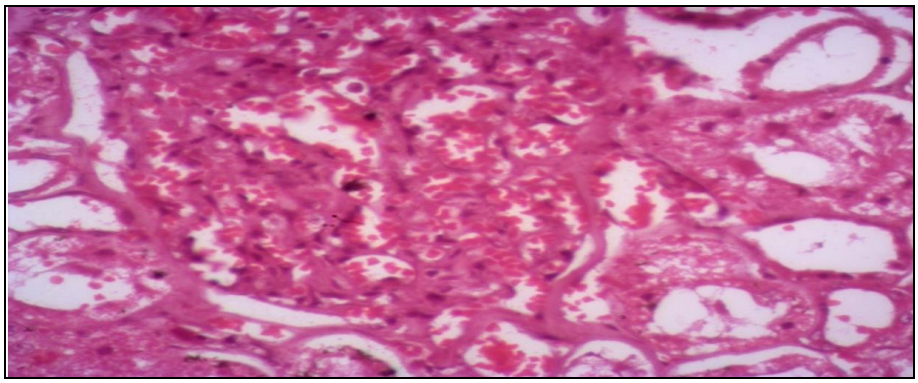

Fig 7: Kidney microscopic 40x image, showing the Glomerulonephritis and Micro-Haemorrhage

On cultural examination the samples didn't show growth of any pathogenic organisms on Blood agar even after 48 hours of incubation. EMJH semisolid media has shown the growth of Dingers ringer's ring after two weeks. On observation under dark field microscopy motile (cork screw movement) leptospira organisms was noticed. On examination in 2% agarose gel of the PCR products of kidney and heart sample have yielded the amplicon size of 285bp (G1&G2) primers and 756bp (*LipL32*) primers, indicating that, the given sample was positive for leptospirosis. The G1&G2 primers derived from the house keeping partial *secY* gene (coding for Translocase preprotein function) and conserved in almost all serogroups. The PCR with G1&G2 primers can detect all serogroups / serovars except *grippotyphosa* (Cheema *et al*., 2007) [10]. Therefore, the samples were also subjected to *LipL32* specific PCR. The surface outer membrane macromolecules are highly conserved in *LipL32* gene which can detect all pathogenic serogroups / serovars including *grippotyphosa* and can be efficiently utilized for diagnostic purpose (Meenambigai *et al*., 2011) [6].

On histopathological examination, the kidney showed interstitial nephritis that consisted of inflammatory infiltrate with predominance of lympho-plasmocitary cells and histiocytes with diffuse and focal distribution (Agudelo-Flórez *et al*., 2013) [1]. Besides, glomerulonephritis. Lung and Liver didn't show much changes.

4. Discussion

Thus, based on PCR, Gross Pathology and Histopathological studies it was concluded that the Hyaena has died due to Leptospirosis. The present findings were in concurrence with the findings of Andhra Pradesh – Tirupathi National Park studies (Rani Prameela *et al*., 2021) [9]. In that study they have reported the seroprevalence of Leptospira antibodies in Hyena in SV Zoological Park. Several research workers *viz*., (Hartskeerl & Terpstra, 1996) [4] and (Pérez-Brígido *et al*., 2020) [7], who have also found the seroprevalence of leptospiral antibodies in different wild life species. In the present study the Hyena might have picked up the infection mainly from rodents which acts as reservoir for leptospires but to establish the linkage we need to take up a detailed study in different wild life species in the zoological garden along with rodents using rodent traps. Serosurvey of the captive wild life species of the National Park might clearly tell us the status of exposure or reservoir cases.

5. Conclusion

In the present study based on the laboratory investigations it can be concluded that the Hyena has died due to Leptospirosis. To ascertain the possible source for spread of the infection we need to carry out the sero survey of other animals present the park for Antigen / Antibody prevalence.

6. References

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