ISSN: 2356-7767

Trials for isolation,molecular typing and serodiagnosis of Leptospira infection in African Catfish Clarias gariepinus

***Shereen, S. Moustafa; *Hanem, A. Mahoud; **Dina, M. Mansour and ***Doaa, M. Gad**

*Animal Health Research Institute (AHRI), Mansoura Branch, Bacteriology Dept.
**Animal Health Research Institute (AHRI), Mansoura Branch, Fish Disease Dept.
***Animal Health Research Institute (AHRI), Kfr El-Shiekh Branch Fish Disease Dept.

Received in 13/1/2019
Accepted in 19/2/2019

Abstract

Leptospirosis is an infectious disease caused by Spirochaete in the genus *Leptospira*, its spread more readily in some tropical regions as *Leptospira* can survive longer in warm and humid environment and affect awide range of mammals and humans. Leptospirosis studies in fish are lacking in our country despite favorable environment and reservoirs which can spread Leptospires in aquatic habitats and may infect fish . Eighty blood samples were collected from alive wild catfish (Clarias gariepinus) 21/80 were positive for MAT with a percentage of (26.2%) as six Leptospira serovars which represented as (*L. interrogans, serovar Canicola, L. interrogansserovar, Icteroheamoragica, L. interrogans-serovars Pomona, L. kischneri serovar Grippotyphosa, L. borgpterseni serovar sejroa* (all are pathogenic strains) and *L. biflexa serovar biflexa* (non pathogenic strain), were traced antibodies while *L. interogenes* serovar *Canicola* and *L. kischneri* serovar *Grippotyphosa* which react with high titer 800 IU/ml in 13 fish serum samples with a percentage of (61.9%) for serovar Canicola. 8 fish serum samples reacted with 600IU/ml with a percentage of (38.1%) with serovar Grippotyphosa. Culture of blood samples collected from such catfish on Leptospira medium revealed that from (21) examined samples (blood of samples positive by MAT), 5 samples were positive for isolation with a percentage of (23.8%). In this investigation, Leptospiral DNA was extracted and exposed to molecular typing using PCR for detection of *L. interrogans* rrs (16S) gene, using 1500 bp thermofisher DNA marker ladder. Results of PCR revealed that from five positive samples by isolation (positive for serodiagnosis by MAT) were tested by PCR, two samples were only positive with a percentage of (40.0%).

Keywords: *Leptospira, Catfish, Clarias garipinus, MAT, molecular typing.*

Introduction

Leptospirosis is a zoonotic disease caused by spirochete bacterium of the genus Leptospira that affect humans and animals worldwide (**WHO, 2011**). Leptospires can live longer in water and moist soil with pH 6–8 and have the ability to form biofilms during interaction with environmental bacteria (**Barragan *et al*., 2011**). There are pathogenic, saprophytic and intermediary leptospires found in natural environment and animal hosts**.** Humans get leptospirosis through contaminated environment and /or direct contact with infectious materials such as urine and blood from infected animal. The disease is associated with certain occupational activities such as rice and sugarcane farming, fishing and fish farming, handling animal products and water sports (**Mgode *et***

al*., 2010)** and (**Cutler *et al*., 2010**). Clinical symptoms of leptospirosis in humans are variable (**Faine, 1982**), and may be mistaken with other diseases including malaria. Leptospirosis is widely distributed in African continent that has the highest burden on global scale (**WHO, 2011)**. The high prevalence of leptospirosis in various animal hosts and humans in Africa suggests that this disease could be abundant in aquatic hosts such as freshwater fish, which could maintain the pathogens and disseminate it to humans. Few studies in Europe have reported leptospirosis in fish and fish farmers (**Barragan *et al.,* 2011**). Human leptospirosis cases associated with freshwater environment especially stagnant water and fish pond areas have been reported in Serbia (**Svirčev *et al*., 2009**). Leptospirosis is an acute febrile illness has a world wide distribution but is most common in tropical countries which characterized by clinical features broad ranging but are often similar to those of other infections that commonly coexist in the tropics such as scrub typhus ,dengue fever and malaria (**Vanaporn *et al*., 2007**) . Leptospira are classified into 24 sero-groups and more than 200 serovars according to the difference of their lipopolysaccharide. Currently, *Leptospira* can be genetically classified based on DNA hybridization technique into at least 19 species (**Evangelista and Coburn, 2010**). Cultivation of leptospires requires special media and it takes at least one week before organisms can be observed. Confirmation of leptospirosis diagnosis mostly relies on antibody detection. Microscopic agglutination test (MAT) has been widely used as the reference test for antibody detection. MAT is performed by incubating serum with various serovars of leptospires. MAT titer is obtained by testing various serum dilutions with the positive serovars. The serovar that reacts with patient serum is suggested to be the infecting serovar. Information on infecting serovars obtained by MAT has been used for epidemiological study (**Chintana *et al.*, 2014**). However, serological testing is complicated because of the high degree of cross reactivity between various Leptospira serovars ,recently molecular techniques such as conventional and real time PCR are recognized as specific and sensitive tests for the rapid detection of infection during early stage of the disease and often negate the need for the isolation and culture of infecting organism for a confirmatory result. These techniques can be performed on various templates including blood, urine and kidney tissues (**Sohail *et al*., 2016).** Leptospirosis is an important environmental disease and a major threat to human health causing at least one million clinical infections annually. Recently, there has been a growing interest in understanding the environmental lifestyle of Leptospira ***(Roman et al.,2018). So,the objective of this study was to determine the presence of *Leptospira* organisms in randomly collected blood samples from alive wild catfish *Clarias gariepinus* catching from of small canals were agriculture drainage waste water at Dakahlia Governorate through, isolation, serological and molecular techniques.

Materials and Methods

1- **Fish:** This study was conducted at A.H.R.I, Mansoura laboratory branch: Two hundred and thirty alive catfish (*Clarias gariepinus*) with a mean body weight ranged from of 750 to1100g at the period between May to August 2017.

2- Blood sampling: 80 Blood samples were collected from caudal vein after sterilizing the area used and pooled from 3 live adults per sample (Catfish were rinsed thoroughly in clean water and wiped) ,samples were collected using 2 ml plastic syringes with 22-gauge needles treated with the anti-coagulant ethylene diamine tetra acetate acid (EDTA) for microscopic examination ,culture and PCR and without anticoagulant for serology by MAT.

Table (1). Type of blood samples used for different techniques:-

Type of blood sample	Test
Plasma(from blood with EDTA)	Microscopic examination
Serum	MAT (Microscopic agglutination test)
Whole blood with EDTA	Culture & PCR

2- Microscopic examination by differential centrifugation according to Wolfe (1954): blood specimens were centrifuged at 500r.p.m for 15min-a drop of plasma was examined by dark filed illumination microscopy. The plasma then centrifuged at 1,000 r.p.m for 20min and the sediment examined microscopically.

3- Microscopic Agglutination Test (MAT) For The Detection Of Antibodies to Leptospires (WHO, 2007):-

Reagents:

Leptospira cultures (different serovars); PBS, pH7.2. Leptospira standard strains denoted from Pasteur Institute and Procedures were made in CBRS, Center of Biotechnology Research & Services, Faculty of Veterinary Med., Cairo University)

Table (2). Leptospira standard strains

Species	Serogroup	Serovar	Strain
L.interrogans	*Canicola*	*Canicola*	*Hond Utrecht VI*
L.interrogans	*Icterohaemorragiae*	*Copenhageni*	*Wijnberg*
L.interrogans	*Pomona*	*Pomona*	*Pomona*
L.Kischneri	*Grippotyphosa*	*Grippotyphosa*	*Moskva V*
L.borgpterseni	*Sejroe*	*Sejroe*	*M84*
L.biflexa	*Semaranga*	*Patoc*	*Patoc 1*

Procedures:

The serum samples were screened by MAT using a battery of 6 serovars of Leptospira as shown in table 2. Briefly, five to seven days old liquid culture of live leptospires with a density of approximately 2 X 10^{λ} leptospires per millilitre was used as antigen. The test was carried out in a 96 well 'U' bottom microtitre plate. Serum dilutions were made in 96 well microtitre plates for which 196 µl of PBS was mixed with 4 µl (1:50) of serum. From the1:50 diluted serum sample, 30µl was added to each of the 8 wells in each column of the microtitre plate. In the last column, only 30µl of PBS was added which served as antigen control. Different serovars (30µl) were added in wells in such a way that each row was charged with only type of antigen including the respective antigen control so that the final serum dilution was 1 in 100. Thus, each row corresponded to each serovar for different serum samples. The plates were closed with lid and incubated at 30ºC for two to four hours. A drop (10µl) of the mixture was placed on clean grease free slide and the wet preparation was examined without coverslip using 20X objective of the dark field microscope for the presence of agglutination or reduction in number of organisms in comparison to the respective antigen control.

Method of detection by microscopic agglutination test:

The endpoint (titer) was taken as that dilution which gave 50% agglutination, leaving 50% of the cells free .It was Compared with a control suspension of leptospires diluted 1:2 in PBS without serum.

4-Isolation of Leptospira organisms in culture from blood morphologically according to Vanaporn *et a*l., (2007)

Twenty one (21) positive blood samples for MAT were used for culturing as 100µl whole blood with EDTA was added to 3 ml of EMJH medium (Ellinghusen, McCullough, Johnson and Harris) supplemented with 3% rabbite serum and 0.1% agarose , after centrifugation at 3,000 rpm for 10 min 500µl of surface plasma was carefully removed and placed into another EMJH. Deposit from spun plasma was collected and placed into a new tube which was centrifuged at 6,000 rpm for 3 min, the supernatant was removed to leave 200µl of deposit which was resuspended and placed into EMJH medium. All cultures were incubated aerobically and protected from light at room temp. (25-30ºC) and examined weekly for 3 monthes by placing one drop of culture onto a microscopic glass slide and observing it by dark field illumination.

5– Molecular characterization of Leptospira (Merien *et al*., 1992):

Target gene	Primers sequences 5′–3′	Amplified segment (bp)	Primary denaturation	Amplification (29cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
L. interrogans rrs 16S	LepI,5′ GGCGGCGCGTCTTAAACATG3′ LepII5′ TTCCCCCCATTGAGCAAGATT3′	313	94˚C 3min.	94˚C 1min.	63˚C 1.5min.	72˚C 2min.	72˚C 10 min.	**Merien *et al.*, (1992)**

Preparation of samples for PCR analysis (Extraction of DNA from Culture): serial 10-fold dilutions of leptospires in sterile distilled water were collected by centrifugation at 13,000 x g for 15 min at 4°C. The pellets, washed twice with 100 ,ul of distilled water, were re-suspended in 10 RI of tris-borate EDTA buffer and heated at 96°C for 10 min.. Pellets were used for PCR amplification.

PCR amplification of Leptospira 16S RNAgene: (Merien *et al*.,1992)

Amplification of DNA was performed in a total volume of 50µl. The reaction mixture consisted of 50 mM KCl, 10 mM Tris hydrochloride (pH 8.4), 2 mM MgCl2, 1 mg of gelatin per ml, 1,uM each oligonucleotide primer, and 200 ,M each dATP, dTTP, dCTP, and dGTP. One unit of Taq DNA polymerase (Beckman Instruments, Inc., Fullerton, Calif.) was used. A 1/10-volume sample was then added, and the reaction mixture was overlaid with 50 µlof mineral oil (Sigma). PCR was performed in a DNA thermal cycler (Gene ATAQ Controller; Pharmacia LKB). The first cycle consisted of denaturation at 94°C for 3 min, annealing at 63°C for 1.5 min, and extension at 72°C for 2 min. The next 29 cycles consisted of denaturation at 94°C for 1 min, primer annealing at 63° C for 1.5 min, and extension at 72°C for 2 min (an additional 10 min was included at the end of the cycles to complete extension of the primers).

Detection of PCR products : The amplification products (10 µl or one-fifth of the reaction mixture) were analyzed by electrophoresis in a 1.5% agarose gel (stained with ethidium bromide). 1500 bp molecular size markers used were thermofisher (Boehringer, Mannheim, Germany) of the following size, 1500bp.

Results

1- Resultsof microscopic examination of blood by differential centrifugation according to Wolfe (1954): from 80 blood samples examined under microscope only 9 samples revealed : long spiral actively motile with nu-

merous, small coils with hooked ends as a characteristic morphological feature.

2- Results of Microscopic agglutination test (MAT): a total of 80 blood samples (serum) collected from catfish were screened for Leptospirosis antibodies using six live *Leptospira* serovars, 21 samples were seropositive reacting with Leptospira serovars by MAT with a percentage of 26.2% as shown in table (2).

Table (3). Leptospira positive serum Catfish samples by MAT

Sample No.	Positive	Negative	Prevalence of positive %
80	21	59	26.2

3- Results of prevalence of Leptospira serovars in fish serum samples *L. interogenes* serovar *Canicola* and *L. kischneri* serovar *Grippotyphosa* which react with high titer 800 IU/ml in 13 fish serum samples with a percentage of (61.9%) for serovar Canicola and (8) fish serum samples react with 600IU/ml with a percentage of (38.1%) with serovar Grippotyphosa as shown in tables (3 & 4).

Table (4). Prevalence of different Leptospira Serovars reacting titer in Catfish Claris gariepinus serum samples:-

	Leptospira serovars					
Strains	**L. *interrogans, serovar-Canicola***	***L. interrogans serovar Ictero-heamoragica***	***L. interrogans serovar Pomona***	***L. Kischneri serovar Grippotyphosa***	***L. borgptersenni serovar sejroa***	***L. biflexa serovar biflexa***
Sample reaction	1:800	1:200	1:200	1:600	1:200	1:200

Table (5). Number and titer of sero-positive fish serum samples with pathogenic *Leptospira* serovars :-

	Positive pathogenic Leptospira (titer>400)				**Other**
Strains	***L. interrogans Canicola serovar***		***L. Kischneri serovar Grippotyphosa***		
Number of positive	13/21 (61.9 %)	1:800 \titer = 800 IU	8/21 (38.1 %)	1:600 titer = 600 IU	Low titer < 400 IU

4- Result of isolation of Leptospira from blood :-

Culture of blood samples collected from catfish Clarias gariepinus on *Leptospira* media revealed that from (21) examined samples (blood of samples positive by MAT), (5) samples were positive for isolation with a percentage of (23.8%). Examined film under dark field microscope revealed long spiral actively motile with numerous, small coils with hooked ends as a characteristic morphological feature as showed in photo (1)

Examined film under dark field illumination microscope :

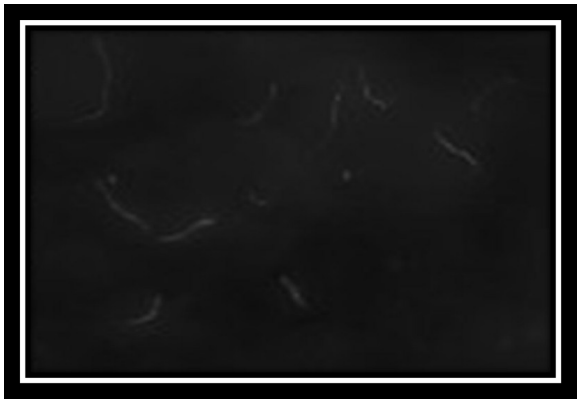


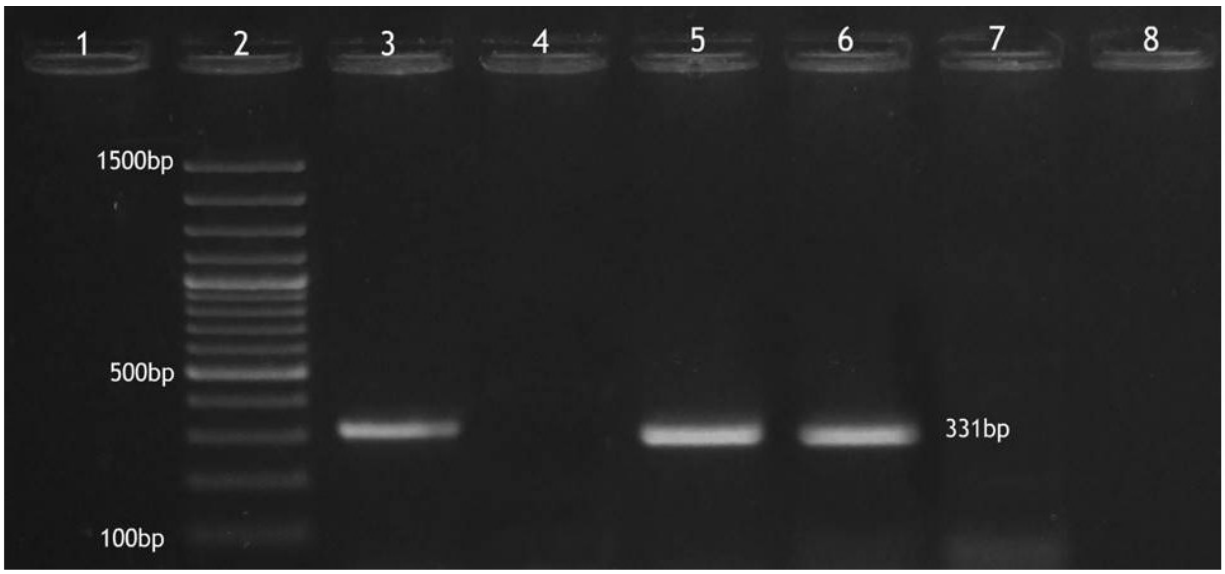
Photo. (1): Examined film under dark field illumination microscope revealed long spiral actively motile with numerous, small coils with hooked ends using the high power objectives (x400).

5- Result of PCR :

Detection of L.interrogans rrs (16S) gene : Five positive samples detected by isolation were tested by PCR after extraction of DNA where confirmed that sample No.5 and 6 were only positive.

Table (6): Result of examined samples by PCR :-

No. of samples	Positive by PCR (rrs(16S) gene	Negative by PCR	%
5	2	3	40.0



6- Electrophoretic profile of L. interrogans rrs (16S) gene; lane (1) negative control, (2) DNA marker ladder 1500 bp (thermofisher), (3) positive control, (5&6) positive sample producing 331bp PCR amplicon

Table (7). Showing the result of Electrophoretic profile of PCR :

Sample	Result
1	Negative control
2	DNA marker ladder 1500
3	Positive control
5 &6	Positive samples

Discussion

African catfish (*Clarias gariepinus*) is one of the most important fish species currently being cultured both inside and outside its natural range of tropical and subtropical environments In tropical countries (**Chepkirui *et al*., 2011**). Leptospirosis is the most widespread zoonotic disease which characterized by non specific clinical signs and its diagnosis is a challenge (**Sohail *et al*., 2016**). In this study, the prevalence of leptospirosis in wild freshwater Catfish catching from small canals in which agriculture drainage waste water in some villages at Dakahlia province, Egypt was investigated. Collected blood samples for culturing ,seroprevalence and genotyping as the presence of Leptospira DNA in cultured fish blood samples were reported to analyse the genetic diversity of Leptospira in the country.Catfish namely *Clarias gariepinus* were randomly collected from area as this species was the predominant and also commonly found in local fish markets. A total of 80 blood samples from such fish were used in this study.

Serological detection of Leptospiral antibodies using microscopic agglutination test (MAT) which is the gold standard test for diagnosis of Leptospira ,MAT detect antibodies against specific causative leptospira serovars with limited ability to also detect antibodies against closely related serovars (**Mgode *et al., 2014***) . Results of MAT revealed that from 80 serum samples collected which were screened for leptospirosis using six live Leptospira serovars (L. *interrogans, serovarCanicola, L. interrogansserovar, Icteroheamoragica, L. interrogans serovar Pomona, L. kischneri serovar Grippotyphosa, L. borgpterseni serovar sejroa* (all are pathogenic strains) and *L. biflexa serovar biflexa* (non pathogenic strain), 21 (26.2%) fish serum samples were sero-positive reacting with Leptospira serovars (*L. interrogans serovar Canicola* and *L kischneri serovar Grippotyphosa*)and showed microscopic agglutination till (1:800) agglutinating titer. The antibody titer 800 IU/ml with *L. interrogans serovar Canicola* and showed microscopic agglutination till (1:600) agglutinating titer and the antibody titer600 IU/ml with *L kischneri serovar Grippotyphosa* with a percentage of (61.9& 38.1) respectively as shown in tables (4&5). Other serovars showed low agglutinating titer which considered as sero-negative. Different serovars were reported by **Mgode *et al.,* (2014)** who detected Leptospira serovars as (25% , 29.2% and 6.3%) for serovars Kenya, Sokoine and serovar Pomona respectively and they mentioned that from 48 fish screened for leptospirosis using four live Leptospira serovars, 26 fish were seropositive reacting with a percentage of (54%) so they detected high prevalence of Leptospirosis in their community. **Magode *et al*., (2014)** also reported that local *Leptospira* serovars Kenya, Sokoine that were the predominant serovars in rodents and cattle in their country which were also the predominant serovars in Tilapia and catfish ,this indicate sharing of Leptospira pathogens between mammals such as rodents ,cattle and aquatic living like fish found in the same locality. Another different results were detected by **Machangu (2006)** who mentioned that the prevalence of leptospirosis in fish is higher exceeding than the prevalence of leptospirosis in terrestrial animals such as cattle and rodents which ranging from 10 to 20% in their country. **Balamurugan *et al.*, (2013)** reported that, preva-

lence of Leptospiral serovars varies depending on particular geographical regions, these explained why there were difference in serovars in different studies.

Blood samples (samples positive for MAT) were collected and used for culture technique into fresh Leptospira EMJH (Ellinghausen and McCullough (1965) modified by Jonson and Harrris) culture media were incubated at (25-30°C) for weeks before results detected. Results of culturing revealed that from 21 examined samples only five samples were positive for isolation with a percentage of (23.8%), Examined film under dark field microscope revealed long spiral actively motile with numerous, small coils with hooked ends as a characteristic morphological feature as shown in photo (1). This result suggested that, diagnosis of leptospirosis through culture is not practical because the complexity of media ,several weeks for growth time and contamination problems in culture media **Erol *et al*., (2015**). Also **Roman *et al.*, (2018**) mentioned that The major difficulty for isolating environmental leptospires resides in the ability to recover a clonal pure *Leptospira* culture from a complex polymicrobial arrangement. Previously, **Ahmed *et al.,* (2005)** mentioned that although all the problems of isolation of Leptospira from clinical samples, it gives a definitive diagnosis also help in identifying the prevalent Serovars. **Sohail *et al*., (2016)** mentioned that, although several techniques have been developed, MAT is still being used for leptospirosis diagnosis and for seroprevalence survey. These data support that MAT could be used for laboratory diagnosis. Four-fold rising of MAT antibody titer is an evidence of *Leptospira* infection. However, Conventional diagnostic tests such as culture and MAT confirm the disease best at a late acute phase in addition to the complexity of reagent in culturing so comparison between above method with molecular typing must be applied. **Roman *et al.,* (2018)** stated that, the diagnostic and surveillance of leptospirosis has mostly used serology and culture for decades. Since the advent of molecular techniques, PCR and real time PCR have been most widely used, PCR is a simple and specific tool for identification of Leptospira in different species. The virulence of *Leptospira* spp. is known to be strain dependent **Adler and Moctezuma, (2010**). So several researchers used 16 s r RNA for identification of pathogenic Leptospira **Prameela *et al*., (2016)** so in this investigation Leptospiral DNA was extracted and exposed to molecular typing using PCR for detection of L.interrogans rrs (16S) gene, using 1500 bp thermofisher DNA marker ladder .Results of PCR revealed that from five positive samples by isolation (positive for serodiagnosis by MAT) were tested by PCR, two samples (No. 5 and 6) were only positive as shown in tables (4 & 5) with a percentage of (40.0%).

Results of molecular detection rate were lower if compared with serological studies. This results agreed with that mentioned by **Picardeau (2013), Sohail *et al*., (2016)** and **Prameela *et al.,* (2016)** who stated that, Molecular detection rate were lower as compared with serological studies because the present study explored blood sample where bacteria are found only after three to ten days of infection but **Roman *et al.,* (2018**) mentioned that comparative analysis of the small ribosomal 16S rRNA subunit gene has been regarded as a standard for bacterial species identification for decades. In *Leptospira*, this gene was also largely used as a target for diagnostics.

Conclusion:

The present research trials to spot light on Leptospirosis in wild Catfish Clarias gariepinus collected from contaminated water sources with special references to molecular identification with aid of microscopic antigenic procedures as serodiagnostic tools where the result indicates the sensitivity of these methods to capture the positive reactivity in such fish.

References

Adler, B. and Moctezuma, A.P. (2010). *Leptospira* and leptospirosis. Vet Microbiol. 2010; 140(3): 287–296.

Ahmed, S.N.; Shahn, S.; Ahmed, F.M.H. (2005). Laboratory diagnosis of Leptospirosis. J. Postgrade Med. 2005, 51: 195-200.

Balamurugan, V.; Gangadhar, N.L.; Mohandoss, N.; Assadi, S.R.; Dhar, T.M.; Shome, R.; Krishnamoorthy, P.; Prabhudas, K. and Rahman, H. (2013). Characterization of Leptospira isolates from animals and humans:phylogentic analysis identifies the prevalence of intermediate species in India. Springer plus 2013. 2, 362 – 370.

Barragan, V.A.; Meji, M.E.; Travez, A.; Zapata, S.; Hartskeerl, R.A.; Haake, D.A. and Trueba, G.A. (2011). Interactions of Leptospira with environmental bacteria from surface water. Current Microbiology 62, 1802 -1806.

Chepkirui-Boit, C.; Ngugi, J. and Bowman, C. (2011). "Growth performance, survival, feed utilization and nutrient utilization of African catfish (Clarias gariepinus) larvae co-fed Artemia and a micro-diet containing freshwater atyid shrimp (Caridina nilotica) during weaning," Aquaculture Nutrition, vol. 17, no. 2, pp. e82–e89, 2011.

Chintana, C.; Rajada, I.; Yong, P. and Duangiai, S. (2014). Interpretation of microscopic agglutination test for diagnosis and seroprevalence. Asian J. 2014, May; 4 (suppl 1) : S162-S164.

Cutler, S.J.; Fooks, A.R. and VanDer Poel, W.H. (2010). Public health threat of new, re -emerging and neglected zoonoses in the industrialized world.Emerging Infectious Diseases. 2010 Jan; 16(1): 1-7.

Ellinghausen, H.C. and Mccullough, W.G. (1965). Nutrition of Leptospira Pomona and growth of 13 other serotypes: fractionation of oleic albumin complex and a medium of bovine albumin and polysorbate80. Am.j. Vet. Res. (26) :45-51.

Erol, E.; Jackson, G.B.; Steinman, M.; Meares, K.; Donahoe, J.; Kelly, N.; Locke, S.; Smith, J.L. and Carter, C.N. (2015). A diagnostic evaluation of real time PCR fluorescent antibody and microscopic agglutination tests in cases of leptospiral abortion. Equine Vet. J, 2015, 47, 171-174.

Evangelista, K.V.; Coburn, J.(2010). Leptospira as an emerging pathogen: a review of its biology, pathogenesis and host immune responses. Future Microbiol. 2010; 5(9): 1413–1425.

Faine, S. (Ed) (1982). Guidelines for the control of Leptospirosis .World Health Organization, Geneva.

Machang'u, R. (2006). Rodent and human disease in Tanzania. RatZooMan Workshop 3-6 May 2006, Malelane, Republic of South Africa, pp 26-27.

Merien, F.; Amouriaux, P.; Perolate, P.; Baranton, G. and Saint, I.G. (1992). Polymerase chain reaction for detection of Leptospira spp. in clinical samples. Journal of Clinical Microbiology, 1992 Sep; 30(9): 2219–2224.

Mgode, G.F.; Machangu, R.S.; Collares, P.M.; Vieira, M.L.; Goris, M.G.A.; Engelbert, M. and Hartskeerl, R.A. (2010). Challenges in determining the pathogenicity status of Leptospira isolates with phenotyping methods: The need for a polyvalent approach. African Journal of Microbiology Reserch 4, 2528 -2533.

Mgode, G.F.; Mbugi, H.A.; Mhamphi, G., Katakweba, A.S. and Michael, T. (2014). Leptospira infections in freshwater fish in Morogoro Tanzania: a hidden public health threat. Tanzania Journal of Health Research. Volume 16, Number 2, April 2014, p 1 -7.

Picardeau, M. (2013). Diagnosis and epidemiology of Leptospirosis. Med. Mal. Infect. 2013, 43, 1-9.

Prameela, D.R.; Sreenivasulu, D.; Seenivasan, N.N.; Vijayachari, P.; Lakshmi, S.V. (2016). 16S RNAPCR for characterization of leptospira from Andhra. pradesh. Int. J Sci. Environ Tech 2016, 5, 84-94.

Roman, T.; Dominique, G.; Emirie, B.; Marie, G.; Anna, R.; Anthony, D.; Michael, M.; Gregorio, I.; Mathieu, P. and Cyrille, G. (2018). Biodiversity of Environmental Leptospira*:* Improving Identification and Revisiting the Diagnosis**.** Front Microbiol**.** 2018; 9: 816. Published online 2018 May 1.

Sohail, M.L.; Khan, M.S.; Avias, M.; Zahoor, M.Y.; Khattak, I.; Ashraf, A. and Nasser, O. (2016). Molecular characterization of Leptospira strains in Pakistan. J. Vet. Res. 60, 417 – 421.

Svirčev, Z.; Marković, S.B.; Vukadinov, J.; Stefan-Mikić, S.; Ruzić, M.;, Doder, R.; Fabri, M.; Canak, G.; Turkulov, V.; Stojanović, D.B. and Draganić, M. (2009). Leptospirosis distribution related to freshwater habitats in the Vojvodina region (Republic of Serbia). Science in China, Series C: Life Sciences 52, 965–971.

Vanaporn, W.; Wirongrong, C.; Direk, L.; Lee, D.S.; Meegan, L.S.; Michael, F.D.; Andrew, T.S.; Roongreung, L.; Yupin, S.; Nicholas, J.W.; Nicholas, P.J.D.; and Sharon, J.P. (2007). Optimization of culture of Leptospira from humans with Leptospirosis. J. Clin. Microbiol. 2007 Apr; 45(4): 1363-1365.

WHO (2011). Report of the second Meeting of Leptospirosis Burden Epidemiology Reference Group. World Health Organization, Geneva, Switzerland.

(WHO) WORLD Health Organization (2007). Leptospirosis laboratory manual, detection disease guidelines laboratory diagnosis of Leptospires, Leptospirosis manual publications/Reports RMRC Port Blair Spirochetes, Delhi English.

WOLFE, J.W. (1954). The laboratory Diagnosis of Leptospirosis. Springfileld: Thomas.