

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

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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 a wide range of mammals and humans. Leptospirosis studies in fish are lacking in our country despite favorable environment and reservoirs which can spread Leptospire 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. interrogans* serovar, *Icteroheamoragica*, *L. interrogans*-serovars *Pomona*, *L. kischneri* serovar *Grippotyphosa*, *L. borgpeterseni* serovar *sejroa* (all are pathogenic strains) and *L. biflexa* serovar *biflexa* (non pathogenic strain), were traced antibodies while *L. interrogans* 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, *Leptospira* 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 gariepinus*, *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). Leptospire 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 leptospire found in natural envi-

ronment 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 varia-

ble (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 leptospire 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 leptospire. 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 tech-

niques 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 to 1100g 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 field 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 Leptospire (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
<i>L.interrogans</i>	<i>Canicola</i>	<i>Canicola</i>	<i>Hond Utrecht VI</i>
<i>L.interrogans</i>	<i>Icterohaemorrhagiae</i>	<i>Copenhageni</i>	<i>Wijnberg</i>
<i>L.interrogans</i>	<i>Pomona</i>	<i>Pomona</i>	<i>Pomona</i>
<i>L.Kischneri</i>	<i>Grippityphosa</i>	<i>Grippityphosa</i>	<i>Moskva V</i>
<i>L.borgpterseni</i>	<i>Sejroe</i>	<i>Sejroe</i>	<i>M84</i>
<i>L.biflexa</i>	<i>Semarang</i>	<i>Patoc</i>	<i>Patoc 1</i>

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 leptospire with a density of approximately 2×10^8 leptospire 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 μ l of PBS was mixed with 4 μ l (1:50) of serum. From the 1:50 diluted serum sample, 30 μ l was added to each of the 8 wells in each column of the microtitre plate. In the last column, only 30 μ l of PBS was added which served as antigen control. Different serovars (30 μ l) 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 30°C for two to four hours. A drop (10 μ l) 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 leptospire diluted 1:2 in PBS without serum.

4-Isolation of Leptospira organisms in culture from blood morphologically according

to Vanaporn *et al.*, (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% rabbit 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 superna-

tant 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. (25-30°C) and examined weekly for 3 months 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		
<i>L. interrogans rrs 16S</i>	LepI,5' GGCGGCGCTCTT AAACATG3'	313	94°C 3min.	94°C 1min.	63°C 1.5min.	72°C 2min.	72°C 10 min.	Merien <i>et al.</i> , (1992)
	LepII,5' TTCCCCCATT- GAGCAAGATT3'							

Preparation of samples for PCR analysis

(Extraction of DNA from Culture): serial 10-fold dilutions of leptospire 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 µl 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,µM each oligonucleotide primer, and 200 µM 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 µl of 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 numerous, 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 react-

ing 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. interrogans* serovar *Canicola* and *L. kirschneri* 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:-

Strains	<i>Leptospira</i> serovars					
	<i>L. interrogans, serovar- Canicola</i>	<i>L. interrogans serovar Ictero-heamorigica</i>	<i>L. interrogans serovar Pomona</i>	<i>L. Kischneri serovar Grippotyphosa</i>	<i>L. borgpferse- ni serovar sejroa</i>	<i>L. biflexa serovar biflexa</i>
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 :-

Strains	Positive pathogenic <i>Leptospira</i> (titer>400)				Other
	<i>L. interrogans Canicola serovar</i>		<i>L. Kischneri serovar Grippotyphosa</i>		
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 :

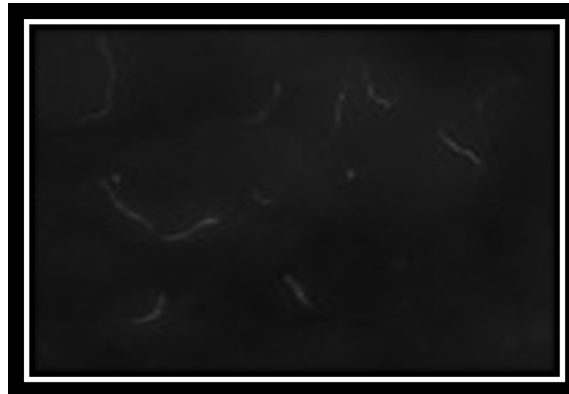


Photo. (1): Examined film under dark field illumination microscope revealed long spiral actively motile with numerous, small coils with hooked ends using the high power objectives (x400).

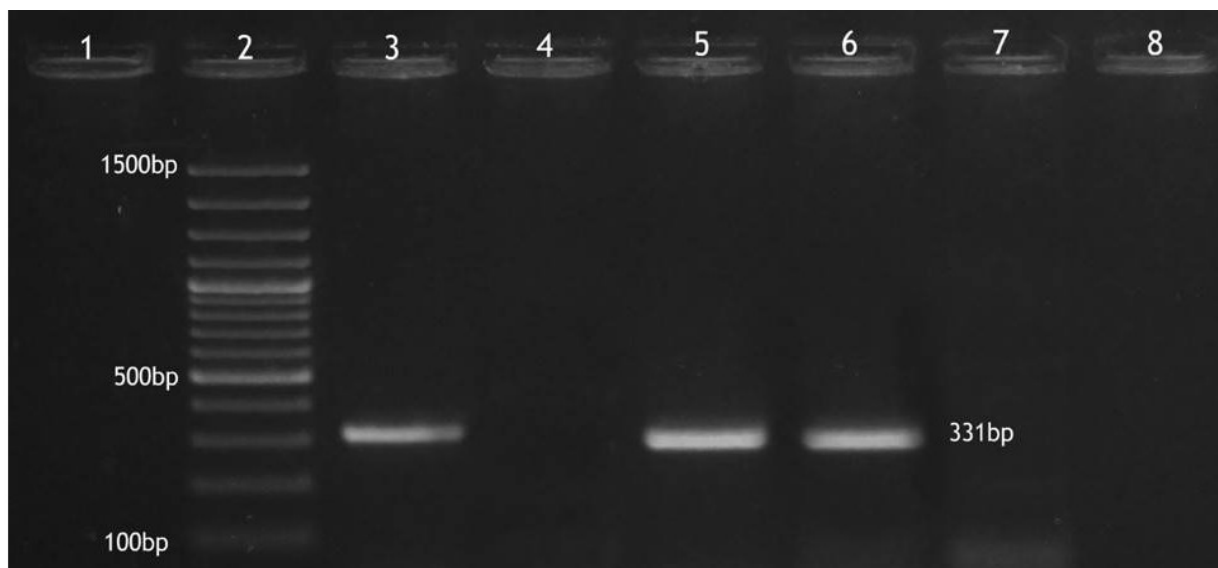
5- Result of PCR :

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 (<i>rrs(16S) gene</i>)	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*, serovar *Canicola*, *L. interrogans* serovar, *Icteroheamoragica*, *L. interrogans* serovar *Pomona*, *L. kirschneri* serovar *Grippotyphosa*, *L. borgpeterseni* 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. kirschneri* 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 titer 600 IU/ml with *L. kirschneri* 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, prevalence 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 Harris) 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.

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