The Value of Polymerase Chain Reaction in the Diagnosis of Tuberculous Meningitis in a Sample of Iraqi Patients

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ABSTRACT:

BACKGROUND:
Tuberculous meningitis (TBM) is a medical emergency. Early diagnosis is of utmost importance to minimize morbidity and mortality. Polymerase chain reaction (PCR) seems to be a promising test for rapid and early diagnosis of TBM.

OBJECTIVE:
To investigate whether PCR detects tubercle bacilli in CSF specimens that are missed by direct microscopy and culture, and if so whether PCR has significant diagnostic value compared to conventional methods.

METHODS:
PCR, culture and acid- fast bacilli (AFB) were performed on CSF samples taken from 43 patients with TBM (based on clinical features and cytochemical parameters of the CSF) and 15 with non- TBM as control group.

RESULTS:
Of the 43 CSF specimens from highly probable TBM patients, 33 were positive by PCR (76.7%), whereas only 5 was acid-fast microscopy (AFM) positive (11.6%) and 22 were culture positive (55.2%). No positive results were found by AFM, culture or PCR in the non-tuberculous control group.

CONCLUSION:
The results of this study indicate that application of PCR is extremely useful for the diagnosis of TBM.The PCR is superior to the currently available techniques for the diagnosis of tuberculous meningitis in terms of sensitivity, specificity and rapidity and can play a critical role in the diagnosis of suspected cases.

KEY WORDS: polymerase chain reaction (pcr), tuberculous meningitis**(**TBM**)**

INTRODUCTION :

Tuberculous meningitis (TBM) is a severe condition, which leads to a fatal outcome. An early and accurate diagnosis and administration of an effective chemotherapy could improve the outcome and reduce the neurological complication[(1)]. Among, extra-pulmonary TB, tuberculous meningitis remains a major health problem in underdeveloped and developing countries [(2)]. Delayed treatment of TBM is associated with high mortality and with neurological problems, which underscores the importance for early diagnosis [(3)].Confirming the clinical suspicion of TBM has always been problematic. Acid-fast bacilli (AFB) staining of cerebrospinal fluid (CSF) has a very low sensitivity [(4)]. Although conventional bacterial culture is the gold standard for diagnosis, the inherent time limitation of the culture based test, limits its value [(5, 6)]. The culture of M. tuberculosis from CSF takes 4–6 weeks and leads to a delay in diagnosis [(7, 8)]. Analysis of CSF using antibody detection is suggestive but not diagnostic of TBM [(9)]. In the absence of any reliable diagnostic methods, various immunological and molecular methods have been advocated including: ELISA [(10)] for demonstration of M. tuberculosis antigen and antibodies, adenosine deaminase assay [(11, 12)], and polymerase chain reaction (PCR) [(13, 14)]. The reliability of PCR depends on the amplification of DNA with primers specific for different target sequences in the mycobacterial genome, and on optimal DNA isolation and PCR procedures [(15)].

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The observed sensitivity and specificity of the PCR for M. tuberculosis in clinical samples differs greatly among the different laboratories ranging from 50–95% and 60–100%, respectively [6].The aim of this study is to investigate whether PCR detects tubercle bacilli in CSF specimens that are missed by direct microscopy and culture, and if so whether PCR has significant diagnostic value compared to conventional methods. This study is the first one done in Iraq using this type of investigation, and because our country is endemic in tuberculosis, its result is very important for early and accurate diagnosis of this serious illness.

PATIENTS AND METHODS:

This study is a multicentric one conducted in Baghdad, al-Yarmouk and Al- Rafidain Teaching Hospitals for the period from August 2009 to October 2010.A total of 58 patients were included in this study,43 of them with highly probable TBM and 15of non-TBM patients as control group.A written consent from the patient or his relatives was taken from all the patients.

The patient's selection was from highly probable TB meningitis based on clinical and laboratory criteria [16]. The lumber puncture and CSF aspiration was performed under aseptic technique using CSF needle gauged 18-22 in, which introduced at L3 or L4 intervertebral spaces and the patient lying on lateral supine position. Five to ten milliliters of CSF samples were collected in sterile tubes, sent for general examination including appearance, sugar, protein, cell count and deferential. Specific tests were performed including Ziehl-Neelson stain, VDRL, Rose-Bengal test, PCR and culture for Mycobacterium tuberculosis.

All fixed CSF smears were stained by Ziehl-Neelsen(ZN) method for acid-fast bacilli (AFB) using strong carbolfuchsin,acid-alcohol as a decolorant, and methylene blue asthe counter stain. After staining, more than 20 fields of eachsmear were examined carefully by the light microscope using the oil immersion (x100) lens.

All CSF specimens were inoculated onto Lowenstein-Jensen (LJ) media without delay and incubated at 37°C for 6-8 weeks and the slopes were examined weekly. The positive cultures were tested by selected biochemical tests for thedefinitive identification of Mycobacterium tuberculosis.

The PCRwas conducted in Biotechnology Research Center Molecular Biology Department/ Al-Nahrain University. For PCR procedure 5 µl of each prepared CSF specimen was incubated in a 45 µl reaction mixture containing 100µl Tris-HCI(pH 8.3), at 500 KCIµl, 15µlMgCl2, 0.1% gelatin, 1 µl of primer (primer capable of amplifying the gene sequence that encode the 38 KD of M. tuberculosis was selected for amplification , the sequence of the primer is 5`ACCACCGAGCGGTTCGCCTGA-3`:CinnaGen-Iran, Cat no. PR7832), 0.2µl each of deoxynucleotides dATP,dGTP, dCTP and dTTP and 1.25 units of Taq polymerase. The reaction mixtures were covered with 40 µl of sterile mineral oil [17].

A control tube containing no target DNA as a negative control, with another tube containing chromosomal DNA of M.tuberculosis as a positive control was included with every set of tests. Precautions were taken to avoid contamination with extraneous DNA. In order to control for the presence of PCR inhibitors, PCR-negative specimens were retested by using 2µl of the chromosomal DNA in the amplification mixture [18].

The reaction was performed using an Conventional thermal cycler (Eppendorf-Germany). The samples were denatured at 94°C for 5 min and then 30 amplification cycles were performed. Each cycle consisted of denaturation at 94°C for 2 min, annealing of primers at 68°C for 2 min and primer extension at 72°C for 2 min. After the 30th cycle, the extension reaction was continued for another 5 min at 72°C [18].

The presence of the 123-bp amplification product was sought by electrophoresis of 5 µl of the amplified mixture at 60 V for 40 min on an agarose gel (1%).The DNA was stained with ethidium bromied (0.5 µg/ml) and visualized on a 302-nm UV transilluminator. The molecular size marker used in this experiment was 123-bp DNA Ladder.

SPSS V.17 was used statistical analysis. Chi square test for independence used to verify the association between discrete variables. Screening tests were validated using the validity measure (sensitivity, specificity, accuracy, and predictive values) with their 95% confidence intervals. Kappa statistics used to test the agreement between the screening tests to exclude the effect of chance. Findings with P values less than 0.05 were considered significant.

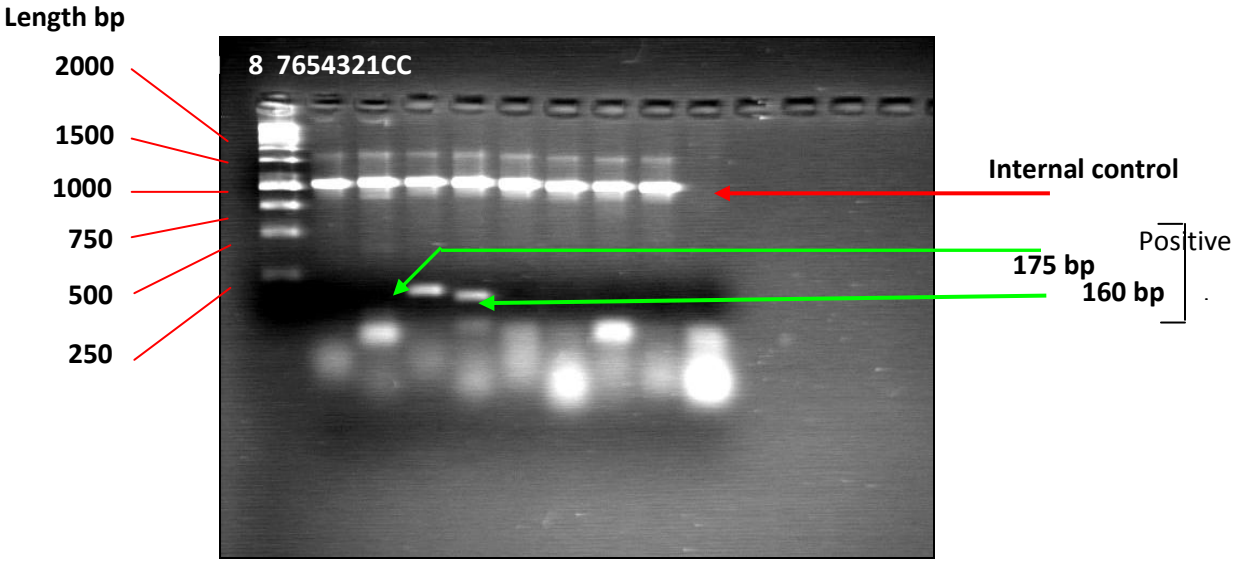

Figure 1 : Electrophoresis was carried out in 2% agarose and stained with Ethidium bromide. Lane M represent DNA Ladder; lane CC represent contamination control. Internal control primers specific for M tuberculosis, Lanes (3, 4) give positive bands.

RESULTS:

The mean age group for cases was 44.02±12.729 and ranging from 12-69 years and it was 40.47±15.647 for control group and ranging from 13-59 years as shown in figure (1).

The sex distribution for the disease group and control group shown in figure (2): 60.47% of cases were males and 39.53% were females while those for control, 53.33% were males and 46.67% were females.

The clinical profile of patients with TBM is summarized in table (1). The first part of the table revealed that the headache is the most prominent complain in 38(88.37%) of the patient followed by fever in 36(83.72%), disturbed sensorium in 26(60.46%) and night sweating in 25(58.13%) of them.Twenty four (55.81%) patients presented with vomiting and lower limb weakness with walking difficulty.Anorexia was significant in about half of the patients 21(48.83%) but history of weight loss was much less frequent and was only in 8(18.60%) of our cases. Generalized ill health and malaise where the disabling complains in 10(23.25%) patients.Seven (16.27%) patients presented with fit(s) and abnormal behaviors.

The second part of this table shows the frequency in order of the presenting signs, meningeal signs were positive in 32(74.44%) of the cases while papilloedema was discovered in 25(58.13%) of the patients.Other cranial nerves abnormalities were variables including facial palsy 15(34.88%), dysphagia 15(34.88%), hearing loss 6(13.95%).Speech, memory and behavioral disturbance were 18(41.86%), 15(34.88%) and 7(16.27%) respectively. Focal signs like hemiparesis 6(13.95%) and sensory impairment 5(11.62%) were not uncommon.

Family history of pulmonary TB was very low 1(2.32%). Fifteen (34.88%) were received BCG vaccine and was proved by the presence of BCG scar in the left arm. Chest X-Ray abnormalities suggested pulmonary TB was found in 8(18.60%) cases. The ESR was elevated in 23(53.48%) patients. Neuroimaging study including CT scan and MRI showed abnormalities suggestive of TBM in 10(23.25%) and14 (32.55%) patients respectively. The above investigations are shown in table (2).

CSF study variables are shown in table and figure (3).The mean CSF protein was 144.60±28.01 (mg/dl) and 74.20±27.40 (mg/dl) for case & control respectively.The CSF sugar was ranging from 25-53mg/dl (M±SD=42.77±8.82mg/dl) for patients with TBM and it ranging from 32-77mg/dl (M±SD=56.87±18.47mg/dl) for those with non-TBM (control).In TBM patients the mean white blood cells was 153.79±67.57 and predominantly lymphocytic pleocytosis (89.02±7.96 %lymphocyte versus 10.98±7.96% neutrophil).The mean WBC count for control was 46.47±62.87 with 72.67±40.480% lymphocyte and 27.33±40.48 neutrophil.

The distribution of participants according to study group and to **screening tests** results is seen in table (4).It revealed that 33(76.7%) out of all CSF specimens with highly probable TBM patients (N=43) were PCR positive and only 10 patients (23.3%) were PCR negative.Twenty two cases (51.1%) were culture positive and the remaining 21(48.8%) were negative by culture. Regarding Z-N stain, only 5(11.6%) patients were smear positive while 38(88.4%) of patients show negative results. All three tests (PCR, Culture & Z-N stain) were negative for control group (N=15). Chi square test was used for statistical analysis and significant result was obtained from both PCR and culture ($p<0.0001$). Non-significant result was obtained from direct microscopy ($p<0.167$).

Table (5) shows thevalidity measures for screening tests with their 95% confidence interval (CI).The sensitivity for PCR, Culture and Z-N stain was 0.77, 0.51 and 0.12 respectively so PCR was the most sensitive test followed by culture. The specificity (with their 95%CI) for all tests was 1.00(0.75-0.99) which means that all tests used were specific. Among tests used PCR was the most accurate one 0.83(0.70-0.91) followed by culture 0.64(0.50-0.76) then direct microscopy 0.34(0.23-0.48).All of the tests were of good predictive values 1.00, but PCR was the best because it had the least confidence interval (CI) 0.87-1.00. So the PCR is the best test among them (good specificity, accuracy and predictive value of positive results).

Table (6)containsdistribution of study sample according to the agreement between screening tests to label screeners as positive or negative. There is a significant association between the results of all the tests (i.e. our findings have not come by chance). There is excellent agreement only between PCR and culture ($K=0.633$, $P<0.0001$). While the agreement between PCR and Z-N stain is significant ($P<0.042$) but poor ($K=0.133$).

DISCUSSION:

Similar to other type of meningitis, TBM showed male predominance [19, 20] .This study is in agreement with the above studies.The clinical profiles of our patients were symmetrical to previous researches [20, 21]. The prominent presenting symptoms were headache, fever, vomiting, anorexia and disturbed level of consciousness and this is in agreement with Verdon R. et al study and also Traub et al in their study [22, 23]. Meningism and papillodema are the most frequent presenting signs in cases of TBM as it is proved previously by the same above series and the study mentioned by Davey and Mancus [24].Some cases not uncommonly presented with seizure(s), hemiparesis and/or behavioral changes [25, 26, 27].

The typical profile of CSF abnormalities was seen in all cases that had been studied. The protein level in TBM was >45mg/dl in all patients and this was proved by many studies conducted before [25, 27].The sugar level was decreased in all patients with TBM and this agrees with the result of other study [27, 28].The WBCs count and differential was the most significant value in differentiating between the pyogenic meningitis and TBM and to lesser extent fromaseptic meningitis, but the difficulty is with partially treated pyogenic meningitis and this was shown also in other studies [21, 29].

The family history of TB in patient with TBM was not significant in this study. The same applied for history of BCG vaccination.

Thirty three of the 43 TBM samples were positive by PCR (sensitivity 77 %) while all the controls were negative (specificity 100 %). Our results show agreement with some previous studies by Ahuja et al. [16], Lin et al. [30], Shankar et al [31], Folgueira et al[32], Machoda et al. [33] while it is much superior to various other studies: Bonington et al. [4], Kox et al. [34], Miorner et al. [21] and Nguyen et al. [35].The reason for lower sensitivity in many studies is unclear. However, it could be due to the low volume of CSF available, inefficient lyses of cells and/or loss of DNA during purification or different methods used for extraction of DNA and presence of inhibitors of PCR in reaction mix [6].

For the detection of overall TBM cases, the PCR assay was useful in terms of sensitivity (77%) as compared to culture and Z-N stain results showing (51%), (12%) sensitivity respectively. Therefore, it has been found that PCR is a good supportive method for rapid diagnosis of clinically diagnosed TBM, particularly where AFB staining and cultures are negative. Some other studies have also suggested that CSF PCR for M. tuberculosis is more sensitive than AFB staining and culture in cases of clinically suspected TBM that responded to empirical treatment [35]. Generally, the specificity of PCR has been very high. Yet, some authors have reported false positive results [31, 36] .The false positive results may be because of nonviable organisms in high prevalence areas or due to DNA contamination [37]. Nevertheless, no false positivity was observed in the present case. The specificity was ensured by the fact that PCR was negative in the 15 CSF specimens from non-TBM patients these results were similar to Kolk et a1 [38]. Also a correlation of the PCR result with the clinical data

and cytochemical parameters is essential, which was seen in our study.

In the present study, the PCR was found to be a rapid diagnostic technique that has better sensitivity than smear and culture with a high degree of specificity for the detection of Mycobacterium tuberculosis complex group of organisms directly from CSF specimens.

In conclusionthe conventional bacteriological methods rarely detect Mycobacterium tuberculosis in CSF and are of limited use in diagnosis of (TBM). The diagnosis of TBM is mainly clinical and paraclinical (including PCR) in addition to high index of suspicion plus the liberal use of spinal needle. PCR is a good supportive method for rapid diagnosis of clinically diagnosed TBM, particularly where AFB staining and cultures are negative. PCR has better sensitivity than smear and culture and a high degree of specificity for the detection of TB bacilli from CSF.

Our study made it evident thatPCR is a rapid and cost-effective diagnostic test for TBM that shows good sensitivity and specificity. This can be adopted as a method of choice for the diagnosis of mycobacterial infections in cases where suspicion is high, in combination with other clinical criteria. Over reliance on PCR should be avoided, as premature cessation of treatment will have serious consequence in patient with TBM, in whom PCR is negative. Hence combination of clinical criteria and PCR is needed in the diagnosis of TBM.Culture should be used concurrently for drug sensitivity testing.

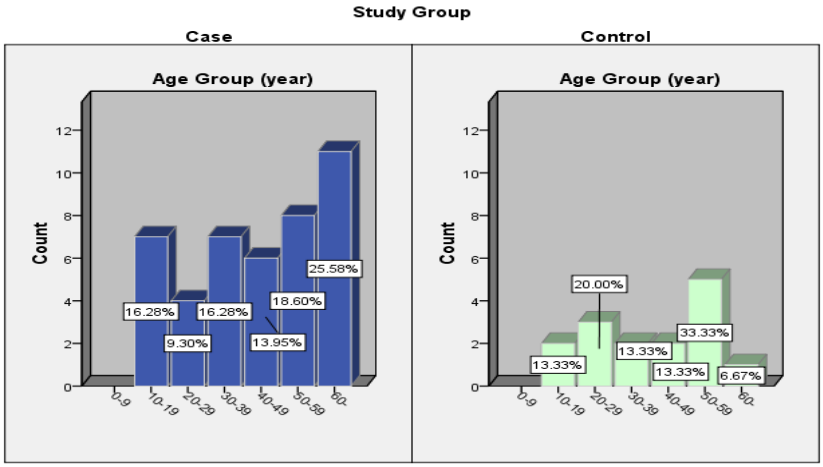

Figure 2: Age distribution in each group of the study sample.

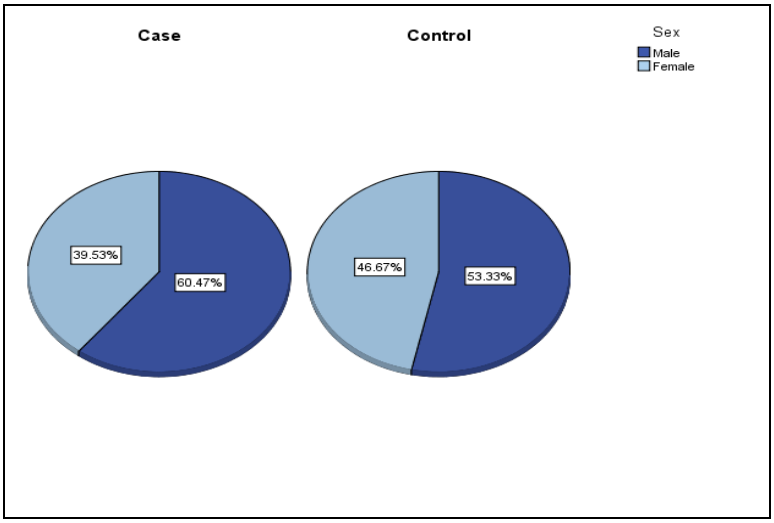

Figure 3: The sex distribution for the disease and control groups.

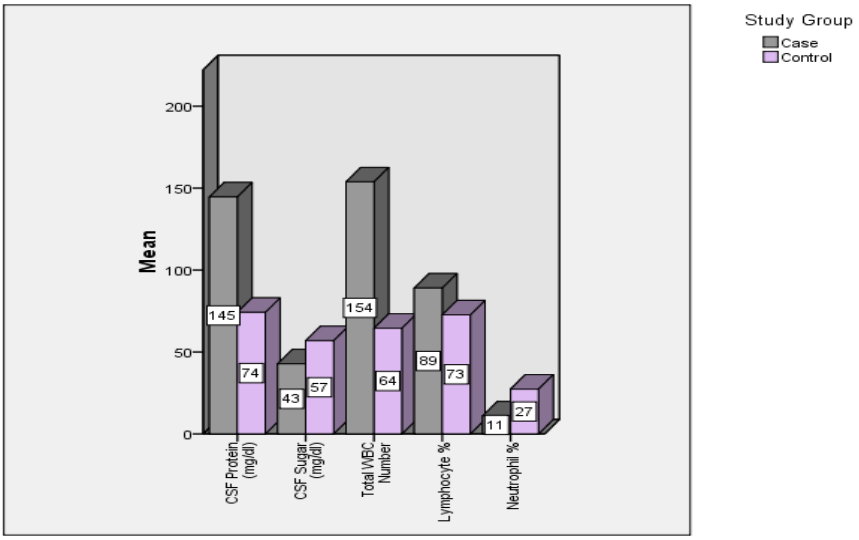

Figure 4: Mean CSF findings according to study group

Table 1: Signs and Symptoms of TBM patients

Signs and symptoms	No.	%
Headache	38	88.37
Fever	36	83.72
Meningeal signs	32	74.44
Disturbed consciousness	26	60.46
Papillodema	25	58.13
Sweating	25	58.13
Gait disturbance	24	55.81
Vomiting	24	55.81
Anorexia	21	48.83
Speech difficulty	18	41.86
Incontinence	18	41.86
Facial palsy	15	34.88
Dysphagia	15	34.88
Memory disturbances	15	34.88
Malaise	10	23.25
Paraplegia	10	23.25
Weight loss	8	18.60
Fit(s)	7	16.27
Abnormal behavior	7	16.27
Hearing difficulty	6	13.95
Hemiparesis	6	13.95
Sensory impairment	5	11.62

Table 2: The different clinical parameters in patients with TBM

History and investigation	No.	(%)
B.C.G. vaccination	15	34.88
ESR	23	53.48
Family history of TB	1	2.32
CXR. Suggestive of pulmonary TB	8	18.60
CT Scan suggestive of TBM	10	23.25
MRI Suggestive of TBM	14	32.55

Table 3: Mean and standard deviations (SD) for different CSF study variables.

	Cases of TBM (N =43)	Control (N=15)
Variables	M±SD	M±SD
CSF Findings		
CSF Protein (mg/dl)	144.60±28.011	74.20±27.400
CSF Sugar (mg/dl)	42.77±8.823	56.87±18.477
WBC total number	153.79±67.570	64.47±62.876
Lymphocyte %	89.02±7.960	72.67±40.480
Neutrophil %	10.98±7.960	27.33±40.480

Table 4: Distribution of participants according to study group and to screening tests results.

		Case	Control	Total	X^2	P
		N (%)	N (%)	N (%)		
		43(100.0)	15(100.0)	58(100.0)		
PCR	Positive	33(76.7)	0 (0.0)	33(56.9)	26.707	0.000
	Negative	10(23.3)	15(100.0)	25(43.1)		
Culture	Positive	22(51.2)	0(0.0)	22(37.9)	12.364	0.000
	Negative	21(48.8)	15(100.0)	36(62.1)		
Z-N Stain	Positive	5(11.6)	0(0.0)	5(8.6)	1.909	0.167
	Negative	38(88.4)	15(100.0)	53(91.4)		

Table 5: Validity measures for screening tests (with their 95%CI).

Validity Measures (95% CI)	PCR	Culture	Z-N Stain
Sensitivity	0.77 (0.61-0.88)	0.51 (0.36-0.66)	0.12 (0.04-0.26)
Specificity	1.00 (0.75-0.99)	1.00 (0.75-0.99)	1.00 (0.75-0.99)
Accuracy	0.83 (0.70-0.91)	0.64 (0.50-0.76)	0.34 (0.23-0.48)
Predictive Value of Positive Result	1.00 (0.87-1.00)	1.00 (0.82-1.00)	1.00 (0.46-0.98)
Predictive Value of Negative Result	0.60 (0.39-0.70)	0.42 (0.26-0.59)	0.28(0.17-0.43)

Table 6: Distribution of study sample according to the agreement between screening tests to label screeners as positive or negative.

		PCR						
		Positive N(%)	Negative N(%)	Total N(%)	X^2	P	K	P
Culture	Positive	22(37.9)	0(0.0)	22(37.9)				
	Negative	11(19.0)	25(43.1)	36(62.1)	26.852	0.000	0.633	0.000
	Total	33(56.9)	25(43.1)	58(100.0)				
		PCR						
		Positive N(%)	Negative N(%)	Total N(%)				
Z-N Stain	Positive	5(8.6)	0(0.0)	5(8.6)				
	Negative	28(48.3)	25(43.1)	53(91.4)	4.145	0.042	0.133	0.042
	Total	33(56.9)	25(43.1)	58(100.0)				

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