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Research Article

Evaluation of Newly Modified Potassium Nitrate Containing Media in Detection and Antibiogram of *Mycobacterium tuberculosis*

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Abstract

Background: *Mycobacterium* is a genus of *Actinobacteria*, given its own family, the *Mycobacteriaceae*. The genus includes pathogens known to cause serious diseases in mammals, including tuberculosis (*Mycobacterium tuberculosis*) and the classic Hansen's strain of leprosy (*Mycobacterium leprae*) [1]. Tuberculosis is the most common life-threatening opportunistic infection (OI) and *AIDS* defining illness in developing countries [2]. The routine identification methods of tuberculosis includes, Tuberculin skin test, Chest X-ray, Growth and physical characteristics of the bacteria, automated based system such as BACTEC 460 TB method, Mycobacterium growth indicator tube (MGIT) method, MB/BacT system, as well as molecular based techniques as in Amplification of nucleic acid for detection of *Mycobacterium tuberculosis* [3-6].

Objectives: To evaluate the validity of newly modified potassium nitrate containing medium (NMK) for rapid detection and antibiogram of M tuberculosis.

Material and method: This was a health facility based observational diagnostics retrospective study. 125 sputum specimens were investigated to evaluate the validity of newly modified potassium nitrate containing medium for rapid detection and antibiogram of M tuberculosis.

Results: 83/125 (66.4%) showed growth after 5-9 days with a mean of 6.58± 0.977 days NMK - containing medium.

Conclusions: The NMK - containing medium showed promising results, it showed a positive growth sings and susceptibility results within 5-9 days of inoculation showing much superior than the golden standard LJ medium.

***Keywords:** Mycobacterium tuberculosis, NMK - containing medium, Antibiogram*

Introduction

The Greek prefix myco means fungus,) Mycobacterium *tuberculosis* is a pathogenic bacterium species in the genus *Mycobacterium*, discovered in 1882 by Robert Koch [7]. *Mycobacterium tuberculosis* is the causative agent of the famous infectious disease; tuberculosis (TB), which usually causes infection to the lungs (pulmonary tuberculosis), but it can also infect any part of the body such as the kidney, bone, spine and the brain [8]. Tuberculosis is one of the most leading causes of death worldwide. The disease affects 1.8 billion people /year, which is equal to one-third of the entire world population [8].

Mycobacterium tuberculosis is aerobic, non-motile, facultative intracellular bacteria that are characteristically acid-alcohol fast [8]. It divides every 16 to 20 hours, an extremely slow rate compared with other bacteria, which usually divide in less than an hour [9].About 25% - 65% of patients with *HIV*/AIDS are with tuberculosis of any organ [11]. TB accounts for about 13% of all *HIV*-related deaths worldwide [10,11]. The TB epidemic is an outgrowth of a long-standing war, which has resulted in poverty, malnutrition, and a large number of displaced populations and refugees. Destruction of health infrastructure, lack of microscopic services, and displacement or lack of health personnel have also contributed to the epidemic [12]. Global tuberculosis epidemic remains a problem for public health, and there is also an emergence of multidrug-resistant tuberculosis that is worsening the impact of this disease [12]. In order to fight this situation, a rapid and inexpensive cultivation and drug susceptibility test media are needed to allow a prompt initiation of correct antibiotic therapy. The X-ray appearance and clinical sign and symptoms are the most common diagnostic methods according to WHO guideline of TB diagnosis in the developing countries [13], defiantly its cheap and inexpensive but it's actually not specific, The second common indicator for tuberculosis was tuberculin skin test, this is also not specific. Sputum smear examination using ZN method remains one of the most common methods of detecting TB but it's usually revealed a false positive results. The definitive diagnosis of tuberculosis depends on the isolation and identification of *M. tuberculosis*. Culture remains the gold standard diagnostic method for tuberculosis, it is a specific and sensitive proved process that is necessarily lengthy because of the slow growth of *M. tuberculosis*, which requires weeks before a positive culture can be identified.

Material and Method

This was a health facility based observational diagnostics retrospective study to evaluate the validity of newly modified potassium nitrate containing medium for rapid detection and antibiogram of *M tuberculosis* comparing to gold stagnated methods.Samples were collected from; Khartoum Teaching Hospital (El-Shaab Hospital) and Aboanga Centre for Respiratory Diseases (Belong to Omdurman Teaching Hospital). Suspected TB patients attending to the above-mentioned hospitals, each of them was instructed to collect early morning with deep coughing sputum (three Sputum samples). Sputum samples were obtained in wide mouth containers. A total number of 125 TB suspects' patients were enrolled in this study. Study was approved by ethical committee –ministry of health - Sudan, and permission was taken from authorities of the both hospitals. The patients included in this study were informed about the study objectives and its importance. All individual in this study did not expose to any dangers during any part of work. Verbal consent was taken and recorded on patient's data sheet. Confidential were granted. Data related to study such as gender, age, tuberculosis history act, were collected by direct interviewing to the patient or his/her relatives using instructed questionnaire. Data was analyzed by calculation of the frequency. Specificity, sensitivity, positive and negative predictive values were calculated.

Laboratory methods

The present study had come out through three phases:

Phase one includes isolation of *Mycobacterium* tuberculosis from the positive ZN sputum samples of suspected patients using routine standard convention method (LJ Media). Then in phase two the samples showed growth were used to evaluate the efficiency of the newly modified potassium nitrate contains media (NMPNM) media in term of ability to grow, rapidity and performance of susceptibility tests. The last phase involves extraction and implication of DNA from isolated strains to identify the isolates. Approximately 1ml of the liquefied sputum was added to 0.5ml of 4% sodium hydroxides (NaOH). The mixture was vortexes and allowed to stand for 15 min (not more than 20 min), then 50ml of Phosphate buffer was added to the mixture [14], and centrifugation at 3000g for 20 min after which the supernatant was decanted into concentrated phenol disinfectant carefully to minimize aerosol generation. *E. coli* and decontamination reagents were simultaneously processed to serve as negative controls and to control contamination, respectively. The pellet was resuspended in 7ml phosphate buffer slain, mixed well and distributed into six containers.

Ziehl Nielsen Stain: One drop, equivalent to 100 µl of decontaminated suspension, was dispensed into a slide which was irradiated under UV light on a hot plate for a minimum of 30 minutes. This was followed by Ziehl Nielsen staining the slides; briefly the slides were flooded with 0.3% Carbol Fuchsin, and flamed using a rod with cotton wool dipped in 70% alcohol for 5 minutes with avoidance of boiling.

The process was repeated three times after which the slides were rinsed with tap water and then it was decolorized with 3% acid alcohol for 2 minutes or till the smear color convert to faint purple, then it were rinsed with tap water to stop the action of decolorization, followed by counter staining with methylene blue for 2 minutes. The slides were rinsed with tap water and allowed to air dry. Examination for acid fast bacilli that appeared red was done under oil immersion at 100 x. Quality control slides were performed using *M. tuberculosis* H37Rv as a positive control and *E. coli* as a negative control and these controls were processed in the same way as the specimen slides. The samples that were microscopically positive with more than 10 acid-fast bacilli (AFB) per microscopic field (scored as "+++") were inculcated into lyses blood agar and Macconky agar. In order to determine the decontamination efficiently and to exclude the non-pulmonary rapid grower bacteria, positive ZN samples were cultured in lyses blood agar and Macconky ager. Commercially LJ media were used in this study (Hi Media), and were prepared as per structure (Appendix- 2). Two loops full of the decontaminated sputum deposit was inoculated on the entire surface of 2 LJ slopes in a pre-sterilized inoculation hood, taking the necessary aseptic precautions. The date of the inoculation was noted and the slopes were incubated at 37 °C aerobically for a maximum period of 8 weeks. They were inspected daily for growth.

Novel modified potassium nitrate containing media (NM Nitrate Media) for identification and susceptibility test:

This Media was composed of middle brooks 7H9 broth, Calf serum albumin Casitone, Oleic Albumin Dextrose Catalase supplements (OADC), multi human vitamin, Potassium nitrate and glycerol and differ from the other available in depending on Calf serum albumin instead of age based one (Manual) and lack of radio or fluorescent substance that may found in automated methods. Firstly the middle brooks 7H9 broth was prepared as per structure of the manufacture (Appendix 3), 25 ml (10.2%)of Calf serum albumin which is main nutrition source, then 20 ml (10%) of Oleic Albumin Dextrose Catalase supplements (OADC) was added to the prepared 7H9 which act as source of protein and sort of selectivity, 1ml (0.5%) of sterile glycerol which acts to prevent the dryness of media and as source of carbon added and mixed well, then 1,000 µg/ml of potassium nitrate was added as growth indicative agent and the bottle was marked with ingredient, stability date and name of the producer and left in the incubator at 37°C for one night. In the next morning, the prepared media were checked for sterility. Inculcation of novel media were performed by adding one ml of sample for each culture medium (1ml / 4 NM K-nitrate free antibiotics media) and incubated aerobically for 5-9 days at 37°C, the medium was then checked for the growth at day five, six, seven, and eight by adding griess solution which develop a weak or strong red colour in positive culture indicating that the nitrate was reduced to nitrite.

Antibiotic susceptibility by direct proportional LJ Medium, NMK - Nitrate medium, and indirectly using resazurin micro titter assay methods.

The first line of tuberculosis antibiotics; Rifampicin, Streptomycin, Isoniazid and Ethambutol were used to perform the susceptibility test.

Direct Antibiotics susceptibility test using LJ media:

LJ media containing antibiotic; 0.2 µg/ml Isoniazid, 20 µg/ml Rifampicin, 4.0 µg /ml Streptomycin, and 3.0 µg /ml Ethambutol was prepared as per structure of the manufacture (Appendix 3) the proportion method of the direct susceptibility test was done. Each sample was inculcated in LJ containing antibiotic medium and incubated aerobically for 4 - 8 week at 37 °C, the antibiotic was considered sensitive if there was no growth after 4-6 week of incubation (Up to 8 week), inoculated medium free antibiotics was used as growth control.

Antibiotics susceptibility test using NMK - Nitrate containing media:

NMK – Nitrate medium containing separately each of the following antibiotic; 0.2 µg/ml Isoniazid, 20 µg/ml Rifampicin, 4.0 µg /ml Streptomycin, and 3.0 µg/ml Ethambutol was prepared, each medium was inoculated with 0.5ml of sputum sample (each set of antibiotic represented by four culture media for each sample). In order to examine the presence or absence of the growth other set (4 culture media) of media free of antibiotic were inoculated with same sample and incubated on the same conditions, these media were checked for the growth if it was positive then the corresponding medium containing antibiotic could checked and the results were record; developing colour indicated resistant, if no colour appear that indicated sensitive agent.

Antibiotics susceptibility test using BACTEC MGIT960R (Becton Dickinson) *Mycobacteria* Growth Indicator Tubes (MGIT) system:

Mycobacteria Growth Indicator Tubes (MGIT) system was done to confirm the direct and indirect proportion-based susceptibility test that was done in this research.

Genomic DNA extractions and amplification of 38 kDa gene:

Genomic DNA was extracted from the prepared inoculums of suspension, via Cetyl-Trimethylamonium Bromide (CTAB) method[15].The genomic DNA was diluted appropriately to (20-50 ng) and used as template in 50 µl PCR reaction using KD1(5'- CCA AGC AAG ATC CCG AGG GCT-3') and KD2 (5'-TTG ATG ATC GGG TAG CCG TCC-3') (Sinacion - Iran PCR TB Kit), targeting 123bp IS6110 insertion sequence of the 38 kDa gen. Briefly; a 50 µl reaction mixture was set by adding 10µL of genomic DNA and 0.8 µL from 2.5U Taq polymerase and 1.0µL of 300µM deoxyribonucleoside to (38.2 µl) PCR master mix tube (Gene Amp PCR core reagent kit supplied by Applied Bio system Roche Company).

The amplification 123bp of 38 kDa gene was done to confirm the growth results and its conducted in Bio - Rad PCR cycler (USA). Each PCR cycle (37 cycles in total) consisted of a 2 min denaturation step at 94°C, followed by a 1 min annealing step at 55°C, and a 1.5 min elongation step at 72°C, with an initial denaturation step at 94°C for 5 min and a final extension step at 72°C for 15 min. PCR products were run on a 0.8 % (w/v) low-melting-point agarose gel in 1X TAE buffer, with a 1kb ladder (BIORON, GmbH, Ludwigshafen, Germany) and visualized with ethidium bromide staining in Gel Documentation System (Alpha-Inotech, USA).

Results

This study involves of 125 TB patients. The majority of study population was male 83/125 (66.4%), (Table 1).

Table 1. Distribution of study population according to the gender and mean of age.

Participants	Frequency	Percentage	Mean/ years
Females	34	27.2%	33.3
Males	83	66.4%	35.5
Children	8	6.40%	10.90
Total	125	100.0%	26.6

The X-Ray reports of the referred participants showed four major features of lung; which were generalized consolidations 7/125 (5.6%), cavities with lung lymphadenopathy 37/125 (29.6%), consolidations on lung only 30/125 (24%) and infiltrates plus cavities in lung with mediastinal or hilar lymphadenopathy 51/125 (40.8%).

The positive ZN samples (125) were subsequently cultured in lysed blood agar, LJ medium and Novel modified potassium nitrate contain medium (NMK - medium), 42/125 (33.6%) samples showed rapid growth on different media; this growth occurred within 3-5 days in lysed blood agar, 7-10 days in LJ and within 3-5 days in NMK containing medium and therefore these samples were excluded.

Table 2. The results of Ziehl Nielson stain.

Zn Stain Results			Positive tuberculosis based on isolation	
Results	**Frequency**	**Percentage**	**Frequency**	**Percentage**
Positive	125	100%	83	66.4 %
Negative	0.00	0.00%	42	33.6%
Total	125	100%	125	100%

Table 3. Growth on NMK – Nitrate contains media description.

NMK – Nitrate contains media Reaction			
Appearance	**Description**	**Frequency**	**Percent**
Whole media developed red	Strong reactions	47	56.6%
Half of the media developed red color	Intermediate reactions	20	24%
Red color developed on the top of media	Weak reactions	13	15.6%
Faint red color developed on the top of media	Very weak reactions	3	3.6%)

Based on the similarity of isolation results using both media NM K-nitrate media and LJ of isolations between, statistical analysis using pairing T test was done to compare between NM K-nitrate media and LJ Media in term of required time; the statistical analysis showed significant strong negative correlation, with t-statistic of (-30.665) and a significance level of P value (0.000).

Table 4. Comparison statistical analysis between NM K-nitrate media and LJ Media in term of required time using pairing T test.

Paired Samples Test								
	Paired Differences					T	Difference	Sig. (2-tailed)
	Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
				Lower	Upper			
Required time	-19.361	5.752	0.631	-20.617	-18.105	-30.665	82	0.000
Paired Samples Test								
	Paired Differences					T	Difference	Sig. (2-tailed)
	Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
				Lower	Upper			
Required time	-19.361	5.752	0.631	-20.617	-18.105	-30.665	82	0.000

Antibiogram to the first line anti tuberculosis drug was done, using LJ medium, NM K-nitrate based medium, the resultant sensitivity patterns were similar; REF displayed the highest rate of resistance 13 (15.7%), followed by INH 7 (8.4%) and ETH 5 (6%) whereas the most sensitive agent was STR 82 (98.8%) followed by PZA 79 (95.2%). The total of mono Rifampicin resistance was 9/83 (10.8%) and the total of mono Isoniazid resistance were 3/83 (1.1%), the total Multi drug *Mycobacterium tuberculosis* (MDR-M*TB*) were 4/83 (1.4%). Furthermore, these results were reconfirmed by comparing to standard MGIT tube, which display same pattern of sensitivity.

Discussion

Tuberculosis is a public health problem in the developing world today and case detection constitutes and important directly observed treatment, short-course (DOTS) strategy according to the WHO. For successful reduction in tuberculosis morbidity and mortality, identification of TB cases in the community and chemotherapy is foremost important.

The X-ray appearance and clinical sign and symptoms are the most common diagnostic methods according to WHO guideline of TB diagnosis in the developing countries [13]. In this study the sensitivity and specificity of X-ray were 100% and 50.0% respectively. However; these figures were varied as reported by Shadia, *et al* [16] and Joshi, *et al*[17], the variability depend on the disease state itself.

The second common indicator for tuberculosis was tuberculin skin test, in this study it showed specificity of 50.3% and sensitivity of 96.51%, this results was in agreement with Nayak*et al*[18], while Hill *et al*, reports showed a high specificity of 98%, the differences in tuberculin skin test results owing to the nature of the test where it depend on the antigen and cellular mediate immune reaction and there for affected by immunization and previous infection.

Sputum smear examination using ZN method remains one of the most common methods of detecting TB. In the present study 44.2% (125/283) of samples were positive for ZN stain, it showed specificity of 83.0% and sensitivity of 100%, which it was agreed with Sawadogo, *et al* [19], and opposed to Hooja, *et al* [20]. Generally false ZN results may a raised from the specimen contain particles that are acid-fast; these particles may sometimes resemble tubercle bacilli, *ie Mycobacteria* other than *M. tuberculosis*, or the precipitate of staining, which hampers reading or occasionally misleads an inexperienced microscopist. However; our result was in accordance with many previous reports [21,22].

The definitive diagnosis of tuberculosis depends on the isolation and identification of *M. tuberculosis*. Culture remains the gold standard diagnostic method for tuberculosis, it is a specific and sensitive proved process that is necessarily lengthy because of the slow growth of *M. tuberculosis*, which requires weeks before a positive culture can be identified. In this study and in order to enhance the growth of TB, the positive growth sputum samples on LJ media were inoculated into NMK -Nitrate contain media which contain Calf serum Albumin, Multi human vitamins and Casitone, that act as additional sources of nutrition, plus Potassium nitrate that made the media special and differential, moreover the NMK - Nitrate contain media reduced the required time from 4-6 weeks in LJ to 5-9 days, which almost similar to the expensive automated procedures.

The NMK Nitrate contain media which developed in our laboratory and evaluated in this study showed 100% specificity and 100% sensitivity comparing to the gold standard media (LJ Media), and both media revealed same results.

This study revealed that, both cultivation methods showed 14.8% of samples were rapid grower organisms indicating *Pseudo tuberculosis* bacteria *(Nocardia* species) which usually had an acid fastness property, thus resist the action of decolorization in ZN process as well as Petroff digestion-decontamination, that make them contribute to ZN false [23].

In contrast to the other conventional culture methods as LJ and 7H11 media, while it cheap and simple, have the major disadvantage of being very slow, which may take 20 – 56 days for diagnosis and farther more four to six weeks after initial culture for drug sensitivity testing [24]. Comparing to automated culture-based methods, like BACTEC 460, which is a radiometric method, provides results in only 5 days but it is costly and requires disposal of radioactive material as well as in the florescent bases *Mycobacteria* growth indicator tube (MGIT) method [45]. Some more rapid culture methods have been developed and are commercially available, most of which are difficult to implement in the field due to the complexity of the technique or the required special equipments as stated by CDC [25].

Newly modified potassium nitrate containing media showed much superior than LJ based media and very much closely when compared to runtime with the results of MGIT and BACTEC 460 TB for detection and antibiogram for *Mycobacterium tuberculosis*. Molecular genetics methods such as the line probe assay are fast but far too expensive to be used in most resource-poor settings and have been developed mainly for RIF susceptibility testing. Colorimetric assays based on the reduction of dyes, *i.e*. Alamar Blue, or 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide, have been tested with some success [26]. However; NMK - Nitrate media showed many draw back; it developed different patterns of color (Strong, intermediate, week and very week), some media showed sort of clotting during incubation, easily to gate contamination and the results depend on visual bases which differ from parson to parson.

Antibiogram to the First line anti tuberculosis drug was performed using direct NM-K-nitrate based medium, direct proportional methods using LJ medium and indirectly using Rezasurine micro titer plate dilution method and using MGIT based test as additional golden stander methods. The results of antibiotics susceptibility test on NMK- Nitrate media, showed that (56.6%) of the positive samples produced strong reactions. The NMK-Nitrate containing media exhibited a sensitivity rate of 100% and specificity of 100% and positive predicted value of 100%, negative predictive value of 100%, the four methods had significant relation with P-value of (0.000) when compared to each other, these results were in alignment with Kumar, *et al* [27], Gupta *et al* [28] and Yap *et al* [29].

Multidrug-resistant (MDR) tuberculosis (TB), defined as resistance to Isoniazid (INH) and Rifampicin (RIF). The emergence of MDR TB highlights the need for drug susceptibility testing (DST), patient management, and drug resistance surveillance. Early diagnosis is essential for starting an effective treatment regimen and reducing its transmission in the population.

Antibiotic susceptibility results of this study displayed identical result between the two examined methods and the two standard methods even in the mono Rifampicin and multi drug resistances (MDR) as confirmed by MGIT results, which were similar to Panaiotov *et al*[30], and Yap B, *et al* [29] reports.

The mono Rifampicin resistant in this study were (3.2%), that much close to the results of Traore *et al,* (4%) [134], and differed from Lisa*et al,* in USA California (0.4%) [31], whereas the total Multi drug resistant *Mycobacterium tuberculosis* (MDR-MTB) were (1.4%) which was closely similar to the anti-tuberculosis drug resistance as reported by WHO fourth global report for Sudan in 2008 [32].

Conclusion

This study evaluates TB diagnostic methods, comparing traditional and new approaches. X-rays showed 100% sensitivity but only 50% specificity. Tuberculin skin tests had high sensitivity (96.51%) but lower specificity (50.3%). Sputum smear examination with the Ziehl-Neelsen (ZN) method was 100% sensitive and 83% specific but faced issues with false positives due to contamination. New NMK-Nitrate media reduced culture time from 4-6 weeks to 5-9 days, showing 100% sensitivity and specificity, similar to advanced methods like MGIT and BACTEC 460. While NMK-Nitrate media has some limitations, it offers a promising, cost-effective alternative. The study emphasizes the need for accurate drug susceptibility testing (DST) for effective management of multidrug-resistant TB (MDR-TB). NMK-Nitrate media performed well in identifying drug resistance, similar to more expensive methods.

Conflict of Interest

The authors declare no conflict of interest.

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