EVALUATION OF DIFFERENT STAINING TECHNIQUES (ZIEHL NEELSEN STAIN, KINYOUN STAIN, MODIFIED COLD STAIN AND FLUOROCHROME STAIN) FOR THE DIAGNOSIS OF PULMONARY TUBERCULOSIS

A

Dissertation

Submitted to the Central Department of Microbiology

Tribhuvan University

In Partial Fulfillment of the Requirements for the Award of the Degree of

Master of Science in Microbiology

(Medical)

By

Deepika Shrestha

Central Department of Microbiology,

Tribhuvan University

Kirtipur, Kathmandu,

Nepal

2006

RECOMMENDATION

This is to certify that **Ms. Deepika shrestha** has worked under our supervision and guidance on the thesis entitled **"Evaluation of different staining techniques (Ziehl Neelsen stain, Kinyoun stain, Modified Cold stain and Fluorochrome stain) for the diagnosis of pulmonary tuberculosis."** To the best of our knowledge this is an original research work of her and has not been submitted for any other degrees.

_____ _____

(Supervisor)
Dr. Shyamal Kumar Bhattacharya
Additional Professor
Department of Microbiology
BPKIHS, Dharan, Nepal
Kathmandu, Nepal

(Supervisor)
Mr. Binod Lekhak
Assistant Professor
Central Department of Microbiology,
Tribhuvan University, Kirtipur

Date: _____

CERTIFICATE OF APPROVAL

On the recommendation of **Dr. Shyamal Kumar Bhattacharya** and **Mr. Binod Lekhak** this dissertation work by **Ms. Deepika Shrestha**, entitled **"Evaluation of different staining techniques (Ziehl Neelsen stain, Kinyoun stain, Modified Cold stain, and Fluorochrome stain) for the diagnosis of pulmonary tuberculosis"** has been approved for the examination and is submitted to Tribhuvan University in partial fulfillment of the requirements for degree of Master of Science in Microbiology (Medical).

_____

Dr. Anjana Singh,

Head of the Department,

Central Department of Microbiology,

Tribhuvan University,

Kirtipur, Kathmandu,Nepal

Date _____

BOARD OF EXAMINERS

Recommended by:

_____

Dr. Shyamal Kumar Bhattacharya
Supervisor

_____

Mr. Binod Lekhak
Supervisor

Approved by: _____

Dr. Anjana Singh
Head of the Department

Examined by: _____

Prof. Dr. Jeevan Bahadur Sherchand

External Examiner

_____

Ms. Shaila Basnet
Internal Examiner

Date _____

ACKNOWLEDGEMENT

It is with great respect and deep sense of gratitude; I owe my sincere indebtedness to my supervisor **Dr. Shyamal Kumar Bhattacharya, Additional Professor,** who generously contributed valuable time and provided his constant support, unfailing encouragement, acute observation, constructive advices and accomplished guidance during entire period of this research work which enable me to carry out this endeavor successfully.

I wish to express most sincere gratitude towards my supervisor **Mr. Binod Lekhak, Assistant Professor** for his constant support, encouragement, constructive suggestion and efficacy which enable me to bring this thesis in present form.

I express my heartiest gratitude to **Dr. Anjana Singh, Head of Central Department of Microbiology** for her constant support, inspiration and encouragement. I would also like to share my eternal respect to **Prof. Sital Basnet and Dr. Prakash Ghimire, Associate Professor** for their innate inspiration, support, and encouragement.

I express my sincerest gratitude and deep respect to **Prof. Renu Dutta, Head** of Department of Microbiology, BPKIHS for her invalueable help and inspirational support during the time of my thesis.

It is an honour on my part to acknowledge profound indeptedness to **Dr. Soumiya Bhattacharya, Additional Professor,** Department of Anatomy, BPKIHS for her encouragement, inspiration and giving me her precious time in bringing this thesis in its final form.

I am sincerely indebted to **Mr. Iswori Poudel, Senior Statistician**, Department of Community Medicine, BPKIHS for giving me his valuable time for statistical analysis and I sincerely thank him for his critical appraisal and thoughtful suggestions. I express my heartful gratitude to **Mr. RM Pandey, Additional Professor**, visiting faculty (BPKIHS), AIIMS, for statistical analysis.

I take this opportunity to express my heartful gratitude to the **faculty teachers** of Central Department of Microbiology, TU and Department of Microbiology, BPKIHS for all their possible help and encouragement they provided me during the time of my study.

I must extend my sincere gratitude towards BPKIHS for giving me to an opportunity to perform thesis on this reputed institute.

I express thanks to the TB technical staffs for their moral support and help during the entire period of my thesis. I am immensely thankful and express my heartiest gratitude to my friends Mr Surendra, Ms Poonam, Ms Sirjana, Mr Bhola Raj and all my friends, colleagues, and well wishers for their moral support and invaluable help they provided during my study.

A very special acknowledgement to **my father, mother, and family members** who always stood by me, gave inner strength and confidence at all the time. No successful event in my life has been possible without their blessings. Finally, I thank **the almighty**, God who has guided me thorough out my life and showed his blessings.

_____

Deepika Shrestha

Date_____

ABSTRACT

Bacteriological examination of sputum is the cornerstone in the diagnosis of pulmonary tuberculosis in the developing world. The risk of spread of infection and emergence of drug resistant strain has created the need of rapid, sensitive and specific diagnostic test. This prospective study was performed in Tuberculosis research laboratory, Department of Microbiology, BPKIHS, Dharan with an objective to evaluate different staining techniques (Ziehl-Neelsen (ZN), Kinyoun, Modified cold (MC), and Fluorochrome) for detection of acid fast bacilli (AFB) for diagnosis of pulmonary tuberculosis

The study was cross-sectional study. All the samples were processed by modified Petroff's method. From each sample four smears were prepared and were stained with four different staining techniques: ZN, Kinyoun, MC, and Fluorochrome respectively. Culture was employed as gold standard for tuberculosis diagnosis.

A total of 1365 specimens from 500 patients were analyzed. 109 patients (21.8%) were diagnosed as having TB by culture. The positive yield found with the staining techniques were 14.2% by ZN, 13.8% by Kinyoun, 14.4% by MC and 17.6% by Fluorochrome staining methods. With reference to culture, sensitivity of ZN, Kinyoun, MC and Fluorochrome were found to be 57.8%, 56%, 59.6%, and 71.6% respectively. The specificity in ZN, Kinyoun and Fluorochrome methods was 98% and that in MC was 98.2%. The positive predictive value of ZN, Kinyoun, MC, Fluorochrome was found to be 88.7%, 88.4%, 90.3% and 90.7% and the negative predictive value of ZN, Kinyoun, MC, Fluorochrome was found to be 89.3%, 88.9%, 89.7% and 92.5% respectively.

This study indicates that the Fluorescent staining method was found most reliable out of the other staining techniques. The MC method also could be viable alternative to ZN and Kinyoun for primary diagnosis of tuberculosis.

Keywords: *M. tuberculosis*, Ziehl-Neelsen, Kinyoun, Modified cold, Fluorochrome stain

LIST OF CONTENTS

		Page no
Title page		i
Recommendation		ii
Certificate of approval		iii
Acknowledgement		v
Abstract		vii
List of abbreviations		viii
List of Tables		xii
List of figures		xiii
List of photographs		xiv
List of appendices		xv
Chapter I - Introduction		1
Chapter II - Objectives of the study		5
Chapter III - Literature review		6-24
3.1	Epidemiology	6
3.2	Historical background	7
3.3	Transmission of tuberculosis	7
3.4	Socio- economic risk factors of tuberculosis	8
3.5	Determinants of pathogenecity and pathogenesis	9
3.6	Acid fast bacilli	10
3.6.1	Sputum smear microscopy	10
3.6.2	Limitation of the study	17
3.7	Diagnostic approach for tuberculosis	18

3.7.1 Culture media 21

3.7.2 BACTEC AFB system 22

3.7.3 Serology 22

3.7.4 Nucleic acid amplification 23

Chapter IV - Materials and Methods 25-31

4.1 Materials and chemicals used 25

4.2 Methodology 25

4.2.1 Settings 25

4.2.2 Types of study 25

4.2.3 Inclusion criteria 25

4.2.4 Exclusion criteria 25

4.2.5 Sample size 26

4.2.6 Sample collection 26

4.2.7 Study methods 26

4.2.7.1 Treatment of sputum 27

4.2.7.1.1 Inoculation and incubation procedures 28

4.2.7.1.2 Culture examination schedule 28

4.2.7.1.3 Observation of cultures 28

4.2.7.1.2 Preparation of smear 28

4.2.7.3 Reporting of TB cases 30

4.3 Statistical analysis 31

4.4 Experimental design 31

Chapter V - Results 32-42

Chapter VI - Discussion 43-49

Chapter VII - Summary and Recommendations 50-52

Chapter VIII – References 53-60

LIST OF TABLES

Page no

Table 1: Age wise and Sex wise distribution of TB cases in the study group 33

Table 2: Evaluation of the Ziehl-Neelsen stain and Culture results for the primary diagnosis of pulmonary tuberculosis 34

Table 3: Evaluation of the Kinyoun stain and Culture results for the primary diagnosis of pulmonary tuberculosis 35

Table 4: Evaluation of the Modified cold stain and Culture results for the primary diagnosis of pulmonary tuberculosis 36

Table 5: Evaluation of the Fluorochrome stain and Culture results for the primary diagnosis of pulmonary tuberculosis 37

Table 6: Correlation between the Kinyoun staining and the Ziehl Neelsen techniques in slide reading of AFB- smear positive and negative 40

Table 7: Correlation between the Modified cold and the Ziehl Neelsen staining techniques in slide reading of AFB- smear positive and negative 40

Table 8: Correlation between the Fluorochrome and the Ziehl Neelsen staining techniques in slide reading of AFB- smear positive and negative 41

LIST OF FIGURES

Figure1: Bar diagram depicting age wise distribution in culture positive cases

Figure 2: False positive result obtained in different staining techniques

Figure 3: False negative result obtained in different staining techniques

Figure 4: True positive obtained in different staining techniques with reference to culture

Figure 5: Percentage of missed diagnosis cases in different staining techniques

Figure 6: Total positive yield obtained by different staining techniques with reference to culture

Figure 7: ROC curve in different staining techniques

Figure 8: ROC curve for Fluorochrome staining technique

Figure 9: ROC curve for Modified cold staining technique

Figure 10: ROC curve for Ziehl Neelsen staining technique

Figure 11: ROC curve for Kinyoun staining technique

LIST OF PHOTOGRAPHS

Photograph 1: Acid fast bacilli in sputum smear (Ziehl Neelsen stain)

Photograph 2: Acid fast bacilli seen in sputum smear (Kinyoun stain)

Photograph 3: Acid fast bacilli in sputum smear (Modified cold stain)

Photograph 4: *M tuberculosis* growth in culture (LJ media)

Photograph 5: Investigator observing acid fast bacilli

Photograph 6: Acid fast bacilli in sputum smear (Fluorochrome stain)

LIST OF APPENDICES

	Page no
Appendix 1: Materials used	i-ii
Appendix 2: Proforma	iii
Appendix 3: Staining reagent and media preparation	iv-vii
Appendix 4: Statistical analysis	viii-x
Appendix 5: Master chart	xi-xx
Appendix 6: Definitions of terminology used in the study	xxi

ABBREVIATION

AFB	Acid fast bacilli
AFB+	Acid fast bacilli positive
AIDS	Acquired Immuno Deficiency Syndrome
AP	Auramine phenol
AR	Auramine Rodamine
ATT	Anti- Tuberculous Treatment
CD	Cluster of differentiation
DAT	Direct Amplification Test
ELISA	Enzyme Linked Immuno Sorbent Assay
EPTB	Extra pulmonary tuberculosis
EZN	Ehrlich Ziehl Neelsen
FDA	Food and Drug Administration
FM	Fluorescence microscopy
FN	False negative
FP	False positive
HIV	Human Immuno Deficiency Virus
IUATLD	International Union Association of tuberculosis and Lung Disease
LJ	Lowensten Jension
MC	Modified Cold
MDR	Multidrug Resistant
MOTT	Mycobacterium other than tuberculosis
NAA	Nucleic Acid Amplification

NaOCl	Sodium Hypochlorite
NPV	Negative predictive value
OPD	Out patient department
PCR	Polymerase Chain Reaction
PPD	Purified Protein Derivatives
PPV	Positive predictive value
PTB	Pulmonary Tuberculosis
ROC	Receiver Operator Characteristic
SAARC	South Asian Association for Regional Cooperation
SNSmear Negative	
SS+	Sputum Smear Positive
TB	Tuberculosis
TBDI	Tuberculosis Diagnostic Initiative
TST	Tuberculin Skin Test
WHO	World Health Organization
ZN	Ziehl Neelsen