"Estimate the effect of *Mycobacteria sp. isolated from clinical samples and its biochemical characterization"*

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Abstract:

Early diagnosis of disease and prompt initiation of treatment is essential for an effective tuberculosis control program especially in areas where there is a movement of TB patients from one area to another dense population. For this study, during the period of October 2016 to February 2019 all the samples were collected. For this study, during the period of October 2016 to February 2019 all the samples were collected. A series of 200 sputum specimens were collected from 91 suspected TB patients (39 patients provided three samples, 31 patients provided two samples and 21 provided only one sample). Out of 200 specimens, 67 were from home and 133 were spot samples. Out of 200, 59 sputum samples from 28 patients were positive for acid fast bacilli. Of 59 positives, 20 were home (34%) and 39 from spot specimens (66%).

Keywords: Diagnosis, Population, Patients, Sputum.

1. Introduction:

Today, there are increasing demands on limited health-care resources. As a result of this, health economics has become an important tool for decision-making at all levels of health-care organizations (**Niemann S, Richter E, *et al.*, 2000**). As asthma and COPD are two of the main chronic diseases worldwide, it is important to get a better understanding of the societal costs for these diseases, both in terms of costs for health-care resources as well as costs for loss of productivity due to the diseases. A number of estimations of the costs of asthma and COPD have been conducted both in Europe and USA (**Gengenbacher M, Kaufmann SH., 2012**). However, there are still many questions, which need an answer, as most of the studies have not taken into account all kind of costs that are involved in these diseases. How high are the costs? What kind of costs appears due to asthma and COPD? Do the costs differ between different severity grades of asthma and COPD? Is the health-related quality of life affected by COPD? Is the quality of life dependent on the severity of the disease? There is still a lack of knowledge of the costs of respiratory diseases, especially costs of COPD, where a great underdiagnosis exists (**Ormerod LP., 2003**).

Asthma is a chronic lung condition that causes recurring episodes of wheezing and other respiratory symptoms. Chronically inflamed airways are hyperresponsive; they become obstructed and airflow is limited when airways are exposed to various provoking factors, such as allergens, irritants, cold air or exercise

(**Flanagan PG, Williams R, *et al*., 1994**). This makes it difficult for the air to move in and out. This narrowing or obstruction can cause one or a combination of symptoms such as wheezing, shortness of breath, chest tightness particularly at night or in the early morning (**Verma A, Dasgupta N, Pande JN., 1995**). The attacks vary in severity and frequency from person to person and within the individuals. The exacerbations of asthma are episodic, but airway inflammation is chronically present. For many patients, medicines must be taken every day to control symptoms, improve lung function, and prevent attacks ((**Katoch VM, Sharma VD., 1997**). The classification of severity of asthma has varied over time and is partly arbitrary. The most recent classification of severity is based on the GINA guidelines (**Singh KK, Nair MD.,1999**). The severity is dependent on a combination of symptoms, lung function and use of medicines Asthma cannot be cured, but can be controlled. The strongest risk factors for developing asthma include a family history of asthma and especially in childhood allergic sensitization. Asthma is a major health problem in society, and the disease seriously affects both children and adults. During the past five decades the prevalence has increased considerably (**Kadival GV *et al,* 2004)**. A similar trend has been found both in studies from Europe and USA (**Narayanan S, *et al,* 2004**). The prevalence of physician-diagnosed asthma in 1990's was about 5 - 9% in the adult population in northern and western Europe with variations between both countries and regions within the countries (**Ahmed N, *et al,* 1994**). This stable increase in the prevalence of asthma during the last decades has also been demonstrated by the OLIN Studies in northern Sweden (**Brisson-Noël A. *et al,* 1989**). It is still unclear why the prevalence of asthma has increased. There are relatively few studies of the incidence of asthma. One study has found that the incidence increased with 20% between 1986 (**Reijula. *et al*, 1991**), while another study showed no clear pattern of changes in incidence (**Torén et al, 1999**). The incidence of asthma among adults in Sweden seems to be about 2/1,000 persons/year according both to the OLIN Studies and the Nordic-Baltic branch, Respiratory Health in Northern Europe (RHINE), of the European Community Respiratory Health Survey (ECRHS) (**Torén *et al*, 2015).**

Mycobacteria is a ubiquitous, rapidly growing, acid fast bacillus (AFB) which can be found in soil, tap water, and shower heads (**Geng E, Kreiswirth B, *et al*.,2005**) Ernest Runyon developed a system of identifying *non-tuberculous mycobacteria* in the 1950s [3]. This included classification based on pigmentation and growth rates (**Perlman DC,*et al.*,2006**). This system of identification is discussed further in the next section. There are

three distinct subspecies of *Mycobacteria*. including *M. abscessus* sb sp. *massiliense*, sb sp. *abscessus*, and sb sp. *bolletii*. (**Pedro-Botet J, *et al.*,2006)**. *Mycobacteria*. is an emerging human pathogen whose impact has grown considerably in the past decade (**Van Cleeff MR, *et al.*, 2005**) *Mycobacteria* have a unique cell wall made up of mycolic acid, peptidoglycan, and arabinogalactan, which likely contributes to their drug resistance and virulence (**Shah NS, Anh MH., 2008**). Clinical strains of *Mycobacteria* can have smooth, rough, or mixed morphology (**Hopewell PC, *et al.*, 2006**) The association of the bacterial morphology and its implication on disease outcome are poorly understood. However, generally a rough morphology predicts a worse clinical outcome due to the cords which interfere with macrophage phagocytosis (**Pai M, Riley LW, *et al.*, 2004**). The smooth morphology associated with glycopeptidolipid (GPL) production confers the ability of these organisms to form bio-films (**Arend SM, Franken WP,*et al.*,2008**). Bio-film formation allows smooth variants of *Mycobacteria* to colonize the environment (**Arend SM, van Meijgaarden KE,*et al.*,2002**).

It is common for patients to have pieces of their lungs resected in order to eradicate the *M. abscessus* (**Bemer P, Palicova F, *et al.*,2002**). Even after successful treatment, many patients will experience relapse of the disease (**Tortoli E, Cichero P,*et al.*,1999**). In one case, a lung transplant patient was diagnosed with subsequent pulmonary *M. abscessus* infection, and was treated successfully. However, one month later this patient developed deep gluteal abscesses caused by relapse of the *M. abscessus* infection, and eventually succumbed to *bronchiolitis obliterans* (**Tortoli E, Cichero P,*et al.*,1999**). Thus, it is important to find more efficacious drug treatment regimens to prevent relapse. We will expand further on the drug resistance of *M. abscessus* (**Brunello F, Fontana R.,2000**). Minimum inhibitory concentrations (MIC) of standard and novel anti-mycobacterial compounds against *M. abscessus* can easily be determined *in vitro* using broth microdilution assays (**Cai L, Kong F, *et al.*, 2009**). However, *in vitro* assays are poor models for infection and immune responses in humans. Furthermore, there is no definitive correlation between drug susceptibility of *M. abscessus in vitro* with drug efficacy in human patients (**Tan W, Wang K, *et al.*,2004**). Thus, it is important to develop a good *in vivo* model of pulmonary or disseminated *M. abscessus* infection in order to study pathogenesis and screen novel anti mycobacterial compounds in an animal before going into clinical trials.

2. Methodology:

2.1.Data Collection:

For the collection of samples, proper medical concern was taken and there was no significant difference in the age, gender or site of disease.

	SGMH patients n = 116 Number (%)	KBTH patients n = 84 Number (%)	P -value
PATIENT AGE			
0-19	17	9	P-0.2971
20-34	56	36	
35-59	31	27	
60+	12	12	
GENDER			
Female	42	31	P-0.2691
Male	74	53	
SITE OF DISEASE			
Extra Pulmonary	10	26	P-0.6651
Pulmonary	30	50	
Pulmonary & Disseminated	76	5	

Table No.1. The age, gender and site of disease of the Sanjay Gandhi Medical Hospital (SGMH) Rewa, Kushabhau Tahkre Hospital Bichiya, Rewa (KBTH) patients.

2.2.Organism Selection:

Clinical sputum specimens collected were first liquefied and decontaminated according to N-acetyl-L-cysteine-sodium hydroxide (NALC-72, NaOH) method in order to avoid bacteria overgrowth which may obstruct the recovery of mycobacterium. Collected respiratory sputum samples were first transferred into a sterile 50 mL conical polypropylene screw cap centrifuge tube. An equal volume of freshly prepared NALC-NaOH solution was added into the tube. The content was mixed by vortexing thoroughly but for not more than 30 seconds. The tube was then incubated for 15 minutes at room temperature. This allows the mucolytic agent and decontaminant to work. Sterile 0.067M phosphate buffer (pH 6.8) was added to the content to dilute it up to

50mL. The buffer aided to cease the action of NaOH and lower the viscosity of the mixture. The tube was then centrifuged at a minimum speed of 3000g for 20 minutes. The supernatant was discarded into disinfectant-containing container. The deposit remaining in the tube was then re-suspended with 2ml phosphate buffer. The suspension was ready for further examination, including AFB smear preparation, culture medium inoculation and nucleic acid extraction.

2.3.Culture

Culture, although not always positive in patients who are treated for TB, is considered the gold-standard for diagnosis. Recent UK data shows that only 56% of notified TB patients were confirmed with a positive culture (Health Protection Agency 2009). The sensitivity of culture is far higher than microscopy and as few as 10 bacilli per milliliter have been detected (Yeager, Jr. et al. 1967). In addition to the increased sensitivity, culture should be performed on all specimens to provide definitive species identification, drug susceptibility testing and genotyping. Following the removal of contaminating non-mycobacterial organisms, the specimen is then cultured.

2.4. Ziehl-Neelsen Staining:

The slide was flooded with Kinyoun's carbofuchsin for 5 minutes. The slide was rinsed with water. Three percentage of acid-alcohol was used to decolorize the smear for 2 minutes, followed by counterstaining with 0.3% aqueous methylene blue for 30 seconds. After counterstaining, the slide was rinsed with water and dried in a 370C incubator. The ZN stained smear were examined under 100X oil immersion objective with light microscope. Positive smears would be scored (negative, trace, +, ++ or +++) depending on the quantity of *bacilli* in the smear.

2.5.Microscopy

The early diagnosis of TB is central to its control. In the poorest parts of the world, where majority of the disease burden lies, the mainstay of diagnosis is microscopy. Microscopy of respiratory samples may be performed using either light microscopy with a Ziehl-Neelsen or Kinyoun stain or fluorescent microscopy with a stain such as auramine-O. Light microscopy is easier and cheaper and is used in the diagnosis of TB in many resource poor settings. In addition, it may be performed in areas with no electricity by using reflected sunlight. Fluorescent microscopy is more sensitive but conventionally requires an expensive fluorescent microscope and

a dark room. The introduction of low-cost fluorescent LED technology has enabled this more sensitive diagnostic tool to be introduced into the areas where it is most needed (**Persing, D. H., Tenover, *et al*. 2010**).

3. Results : During the study we have found these following observation. Table No1 .

Group	No. of specimens N=200	ZN positive	ZN negative
1	56	25	31
2	61	23	38
3	43	5	28
4	50	6	44
Total	200	59	141

Group 1- Sputum (smear with average no. of <10 SEC and >25PC)
Group 2- MSMP (More SEC and more PCs>10 SEC and > 25PC)
Group 3- LSLP (Low SEC and PC<SEC and <25PC)
Group 4- Saliva (smear having >10 SEC and <25PC)

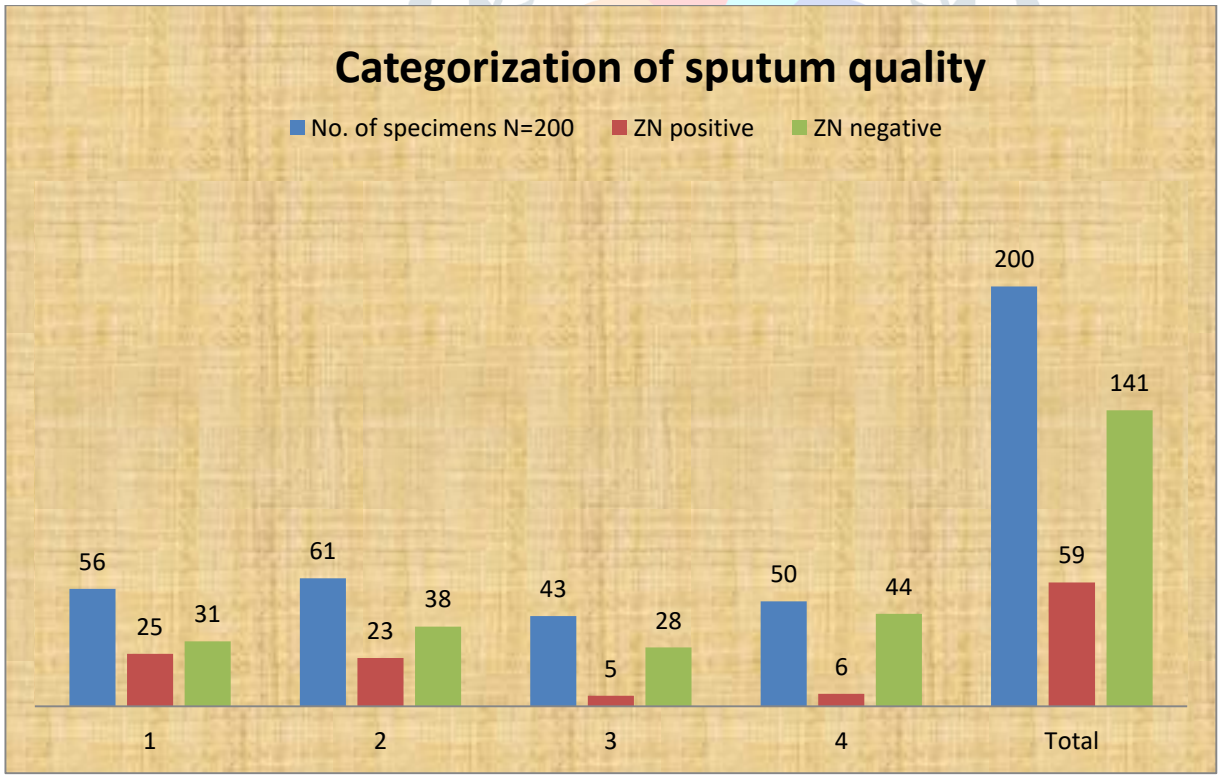

Table No.2. shows ZN test results of isolated microbes.

N=164	ZN Positive N=55	ZN Negative N=109	Grand Total
Number of Pus Cells (PC) (p<0.001)			
<25	11	47	58
>25	44	62	106
Squamous Epithelial Cells (SEC) (p>0.1)			
< 10	28	40	68
>10	27	69	96

>25PC: Associated with ZN smear & culture positivity
>10SEC: Not associated with ZN smear & culture negativity

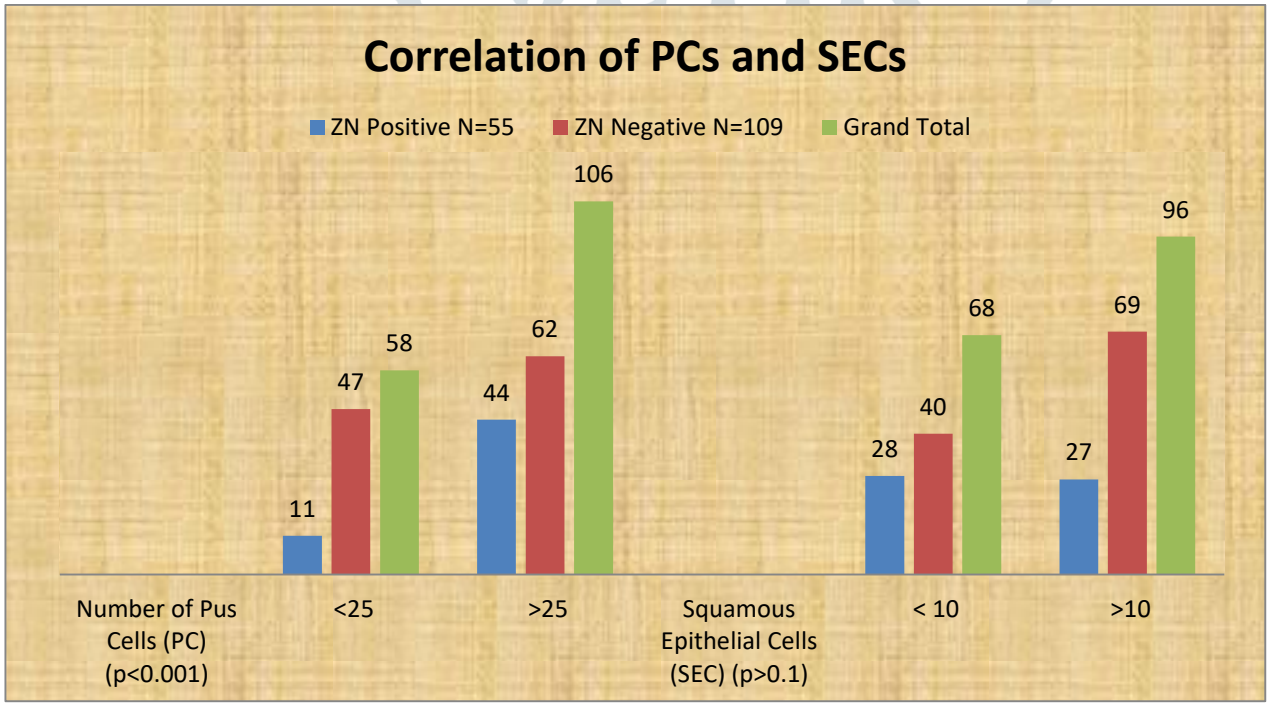

Table No 3: Comparison of results from Culture, ZN Staining & RT PCR Kit methods

METHODS USED			RESULTS					
			ZN STAINING		CULTURE		RT PCR KIT	
			PS	NS	PS	NS	PS	NS
			n=51 (25.5%)	n= 149 (74.5%)	n= 67 (33.5%)	n= 133 (66.5%)	67 (33.5%)	133 (66.5%)
Conventional PCR	**(PS)**	n= 67 (33.5%)	42 (21.0%)	25 (12.5%)	59 (29.5%)	8 (4.0%)	59 (29.5%)	8 (4.0%)
	(NS)	n= 133 (66.5%)	9 (4.5%)	124 (62.0%)	8 (4.0%)	125 (62.5%)	8 (4.0%)	125 (62.5%)

Key: PS(+)-Positive sample , NS (-)-Negative Sample

METHOD USED			RESULTS	
			CULTURE	
			(PS)	(NS)
ZN Staining			n= 67 (33.5%)	n= 133 (66.5%)
	PS)	n= 51 (25.5%)	50 (25.0%)	1 (0.5%)
	NS)	n= 149 (74.5%)	17 (8.5%)	132 (66.0%)

Table No. 4: Comparison of results from ZN Staining with Culture.

3.1. Pathogenicity Determination:

For the diagnose of TB pathogens, we have microscopically observed 200 sputum samples by the help of Ziehl Neelson Stain as **per the guidelines of India's Revised National Tuberculosis Control Programme.** Among from 200samples only the 128 patient's samples were found suitable and from these samples, only 24 patients had initial positive results for AFB in sputum samples. Final cultures for *M.tuberculosis* on Lowenstein-Jensen medium obtained after 6 weeks were positive. TB was confirmed if AFB and/or culture of sputum specimens were positive for *M. tuberculosis*. When both tests were negative, the patients were

diagnosed by clinical symptoms. Sputum samples of 41 TB patients with clinical diagnosis was available but were all negative to culture/AFB staining. All patients received anti-TB drug (not covered under DOT) in absence of any other therapy. Daily dosages were given as isoniazid 300 mg, rifampicin 450 mg or 600 mg, pyrazinamide 1.5 g or 2.0 g and ethambutol 25 mg/kg for the first 2 months. In the next 2 months rifampicin 450 mg or 600 mg, isoniazid 300 mg and ethambutol were given followed by ethambutol, rifampicin 450 mg or 600 mg and isoniazid 300 mg for the next 4 months.

4. Conclusion :

In India TB is one of the major public health problems. About 40% of the total population is infected with TB, of which 60% are in the economically productive age group. Controlling TB is a major challenge in India, where there are several medical hospitals and a good network of Government health care facilities and several private health facilities extending care to TB patients. Tuberculosis is an infectious disease which is transfer by air from one person to other, it has a devastating impact on the economic well being of individual, their families and the entire community. Early diagnosis of disease and prompt initiation of treatment is essential for an effective tuberculosis control program especially in areas where there is a movement of TB patients from one area to another dense population. For this study, during the period of October 2016 to February 2019 all the samples were collected. A series of 200 sputum specimens were collected from 91 suspected TB patients (39 patients provided three samples, 31 patients provided two samples and 21 provided only one sample). Out of 200 specimens, 67 were from home and 133 were spot samples. Out of 200, 59 sputum samples from 28 patients were positive for acid fast bacilli. Of 59 positives, 20 were home (34%) and 39 from spot specimens (66%). When Culture was taken as gold standard, the sensitivity of Conventional PCR assay was 88%, This suggested that among the cases of suspected TB the Conventional PCR assay was more sensitive compared to conventional test of ZN Staining.

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