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Isolation of Bacteria and Yeasts in Sputum Samples of MDR TB Suspected Patients

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ABSTRACT

Introduction

Tuberculosis remains as a major global public health problem and secondary bacterial and fungal infections are one of the most important complications in patients with pulmonary tuberculosis.(1) The present study was conducted to isolate the potentially pathogenic bacteria and yeasts in sputum samples of MDR TB suspects.

Materials and methods

The present study was a hospital based cross-sectional study. After obtaining permission from institutional ethical committee, the study was conducted in the lab of a tertiary health care centre during the month of July 2015.

Results

Out of 76 bacterial isolates 45 (59.3%) were GNB and 31 (40.7%) were GPC. The predominant isolate among GNB was Klebsiella species (48.8%) followed by Pseudomonas species (31.1%), Acinetobacter species (8.8%), *Proteus* species (6.6%) and Serratia (4.4%).

Conclusion

As the prevalence of Candida nonalbicans and Cryptococcus species is increased, routine screening for fungal co-infection is needed.

Keywords

Bacteria, Yeast, multi drug resistant tuberculosis suspects.

INTRODUCTION

Tuberculosis remains as a major global public health problem and secondary bacterial and fungal infections are one of the most important complications in patients with pulmonary tuberculosis.[1] Tuberculosis patients became susceptible to secondary bacterial infections because of many reasons. The major reason is the inhibition of human defence forces during the course of active tuberculosis.[2]

It has also been observed that the alveolar lining material of patients with active pulmonary tuberculosis has less bactericidal activity against bacterial infections. The suppression of human defence mechanisms during the course of active tuberculosis brightens the chances of opportunistic infections. It is not clear whether they are potentially pathogenic micro organisms or only colonization of the respiratory tract but secondary infections caused by resistant organisms like pseudomonas and other drug resistant bacteria and fungi will lead to problems in diagnosis and treatment.[2]

Opportunistic fungal organisms such as Candida species and Cryptococcus species tend to cause diseases in patients who are immunocom promised like in tuberculosis[3]. The reasons for increased prevalence are lowering of immune system due to tuberculosis and the prolonged use of anti tuberculous drugs which promote the growth and reproduction of the fungal flora and in turn aggravate the course of underlying process in the lung tissues.[4] The present study was conducted to isolate the potentially pathogenic bacteria and yeasts in sputum samples of MDR TB suspects.

OBJECTIVES

1. To isolate aerobic bacteria and yeasts in secondary infections from the sputum samples of MDR TB Suspects.
2. To study the antibiogram of Isolated bacterial organisms.
3. To study antifungal susceptibility pattern of the isolated yeasts.

MATERIALS AND METHODS

The present study was a hospital based cross-sectional study. After obtaining permission from institutional ethical committee, the study was conducted in the lab of a tertiary health care centre during the month of July 2015.

Inclusion Criteria

1. 100 Sputum samples of MDR suspects were included in the study.
2. Samples of all the criteria for MDR suspects as per the RNTCP guidelines [5] were included.

Exclusion Criteria

1. Clinically suspected and smear positive new cases were not included.
2. Extra pulmonary cases were not included.

All the samples received at lab were collected in falcon tubes and transported as per the RNTCP guidelines. All the samples were processed for Grams stain, ZN stain and inoculated on Blood agar, MacConkey agar & Sabouraud Dextrose agar (SDA) before proceeding for Line Probe assay (LPA) and MTB culture in liquid medium Middle Brook 7H9 as per RNTCP guidelines. Bacterial growths were isolated from Blood Agar and MacConkey agar after an overnight incubation at 37^0c and identified by biochemical reactions.

Yeasts were isolated from SDA after incubation for 24 to 48 hours at 25^0c and 37^0c, Grams stain was done and for Candida species germ tube test (Reynauldsbraudi phenomenon) was performed to differentiate Candida albicans and non albicans and

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inoculated on Hichrome agar for speciation. For *Cryptococcus* species capsular stain was done with India ink and Urease test was performed.

The antibiotic susceptibility testing was performed by Kirby- Bauer disc diffusion method and zones were measured as per CLSI guidelines [6].Commercially available antibiotic discs (Hi-media) were used.

For GPC the following drugs were used

1. Cefoxitin - 30 mcg
2. Amoxyclav - 20/10 mcg
3. Azithromycin - 30 mcg
4. Ofloxacin - 5 mcg
5. Vancomycin - 30 mcg
6. Linezolid - 30 mcg
7. Meropenem - 10 mcg
8. Ceftazidime - 30 mcg

For GNB the following drug were used

1. Amoxyclav - 20/10 mcg
2. Ciprofloxacin - 5mcg
3. Amikacin - 30mcg
4. Ceftazidime - 30 mcg
5. Ceftriaxone - 30mcg
6. Piperacillin+ Tazobactum - 100/10 mcg
7. Ceftazidime + Clavulonate - 30/10 mcg
8. Meropenem - 10 mcg

E .coli ATCC 25922Pseudomonas aeruginosa ATCC 27853, Staphylococcus aureus 25923 were used as control strains.

Cefoxitin disc was used to detect methicillin resistance in Staphylococcus species and ESBL producing organisms were identified by using ceftazidime and ceftazidime + Clavulinic acid discs as per CLSI guidelines.

Antifungal susceptibility test done by disk diffusion method as per CLSI guidelines M44-A.

Inoculum was prepared from the yeast grown on SDA for 24hrs, adjusted to match the turbidity of 0.5 Mc Farlands standard spectrophotometer. Sterile applicator swab was moistened in that cell suspension and used to inoculate the surface of Mueller-Hinton agar. Mueller-Hinton agar was supplemented with 2% glucose and 0.5µg/ml methylene blue dye medium. Antifungal discs were placed and incubated in BOD for 24hrs to 48 hrs and observed for zones of inhibition.

Antifungal discs used were:

1. Amphotericin-B - 20µg
2. Itraconazole - 10µg
3. Fluconazole - 10µg
4. Ketoconazole - 10µg
5. Clotrimazole - 10µg

For azoles sensitivity zones were

Susceptible – when ≥17mm diameter

Intermediate – in between 14mm-16mm diameter

Resistant – when ≤13mm diameter.

For Amphotericin B sensitivity zones were

Susceptible – when ≥15mm diameter

Intermediate – in between 13mm-14mm diameter

Resistant – when ≤12mm diameter.

RESULTS

Out of 100 samples, 81% were males & 19% females. Majority of the cases were from age group of 30 to 50 years (53%). Out of 100 samples 88 (88%) were culture positive and 12 (12%) were sterile (TABLE 1).Out of 88 culture positive samples , bacteria were isolated in pure from 52(59%)samples and mixed isolates of bacteria and yeasts in 24(27.3%) samples and yeasts were isolated in pure in 12 (13.7%) samples. (TABLE 2)

Total bacterial isolates from pure (52) and mixed (24) were 76. Total number of yeasts from pure (12) and

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mixed (24) were 36 isolates.(TABLE 4)Out of 76 bacterial isolates 45 (59.3%) were GNB and 31 (40.7%) were GPC. The predominant isolate among GNB was *Klebsiella* species (48.8%) followed by *Pseudomonas* species (31.1%), *Acinetobacter*species (8.8%), *Proteus* species (6.6%) and *Serratia* (4.4%). (TABLE 3).

Among GPC, 21 (67.7%) were *Streptococcus pneumoniae* and 10 (32.3%) were *Staphylococcus aureus*. Out of 36 yeast isolates 9(25%) were *Candida albicans*, 21 (58.3%) were *Candida nonalbicans* and 6 (16.7%) were *Cryptococcus* species. Out of total yeasts *Candida tropicalis* was isolated in 12(33.4%),*Candida krusei* in 5(13.8%) and *Candida glabrata* in4(11.12%).(TABLE 5)

The GNB were mostly sensitive to Piperacillin tazobactum, Levofloxacin and Tigecycline. *Pseudomonas* species were 100% sensitive to Colistin.(TABLE 7)

Out of 45 gram negative bacilli 25 were of Enterobacteriaceae, out of which 20(80%) were ESBL producers.*Streptococci* were more sensitive to Imepenem, vancomycin, clindamycin and cefazolin. Out of 10 Staphylococcus aureus isolates 5(50%) were MRSA strains (TABLE 8).The isolated yeasts were mostly sensitive to Amphotericin B(TABLE 9).

Out of 100 samples 54 (54%) were smear positive and 46(46%) were smear negative. Out of 46 smear negative samples 4 samples were positive in liquid culture. Total (54 smear positive+ 4 culture positive) 58 samples were processed for LPA. Out of 58 samples 49(84.48%) were Rifampicin and INH sensitive, 1(1.72%) were Rifampicin resistant and INH sensitive, 4(6.9%) were Rifampicin sensitive and INH resistant and 2(3.45%) were Rifampicin and INH resistant and 2 were MTB not detected.(TABLE 6)

Out of 58 tuberculosis positive samples (54 smear positive & 4 culture positive), 52(89.6%) were coinfected with potentially pathogenic microorganisms and out of 42 smear negative samples 36(85.7%) were coinfected with potentially pathogenic microorganisms.

Table 1: Age Wise and Sex Wise Distribution (n=100)

S.NO	AGE	MALE	FEMALE	TOTAL
1.	10-20 Years	1	2	3
2.	21-30 Years	12	4	16
3.	31-40 Years	20	6	26
4.	41-50 Years	22	5	27
5.	51-60 Years	17	1	18
6.	> 60 Years	9	1	10
	Total	81	19	100

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Table 2: Distribution of Bacterial and Fungal Isolates (n=100)

S.NO		CULTURE POSITIVE	PERCENTAGE	CULTURE STERILE	TOTAL
1.	BACTERIAL ISOLATES	52	59%	12	
2.	BACTERIAL + FUNGAL ISOLATES	24	27.3%		
3.	FUNGAL ISOLATES	12	13.7%		
	TOTAL	88		12	100

Table 3: Distribution of Total Bacterial Isolates (n=76)

S.NO	GRAM NEGATIVE BACILLI	n=45	PERCENTAGE (59.3%)
1.	Klebsiella	22	48.8%
2.	Pseudomonas	14	31.1%
3.	Acinetobacter	4	8.8%
4.	Proteus sps	3	6.6%
5.	Serratia sps	2	4.4%
	Total	45	
S.NO	GRAM POSITIVE COCCI	n=31	PERCENTAGE (40.7%)
1.	Staphylococcus aureus	10	32.3%
2.	Streptococcus pneumonia	21	67.7%
	Total	31	

Table 4: Distribution of Bacterial + Fungal Isolates (n=24)

S.NO		n=24
1.	Klebsiella + Candida albicans	1
2.	Klebsiella+ Candida non albicans	2
3.	Klebsiella + Cryptococcus	1
4.	Klebsiella + Crptococcus	3
5.	Pseudomonas + Candida non albicans	2
6.	Serratia + Candida non albicans	1
7.	Acinetobacter + Candida non albicans	1
8.	Escherichia coli + Candida non albicans	1
9.	Staphylococcus aureus+ C.albicans	2
10.	Staphylococcus aureus+ C. non albicans	3
11.	Staphylococcus aureus+ Cryptococcus	1
12.	Streptococcus pneumoniae+ C. non albicans	3
13.	Streptococcus pneumoniae+ Cryptococcus	3
	Total	24

Table 5: Distribution of Total Fungal Isolates (Yeasts) (N=24+12=36)

S.NO		n=36 (40.9%)	Percentage
1.	Candida albicans	9	25%
2.	Candida tropicalis	12	33.34%
3.	Candida krusei	5	13.8%
4.	Candida glabrata	4	11.12%
5.	Cryptococcus	6	16.7%
	Total	36	

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Table 6: Distribution of LPA results

S.NO		n = 58	PERCENTAGE
1.	Rifampicin & INH sensitive	49	84.48%
2.	Rifampicin resistant & INH sensitive	1	1.72%
3.	Rifampicin sensitive & INH resistant	4	6.9%
4.	Rifampicin & INH resistant	2	3.45%
5.	MTB not detected	2	3.45%
	Total	58	

Table 7 Antibiotic Susceptibility Pattern of Gram Negative Bacilli

S.NO	ISOLATE	LE	MEM	CAC	CAZ	PIT	AK	TGC	CL
1.	*Klebsiella*sps n=22	20 (90.9%)	14 (63.6%)	21 (95.5%)	16 (72.7%)	22 (100%)	14 (63.6%)	18 (81.8%)	NA
2.	*Psuedomonass* ps n=14	12 (85.7%)	12 (85.7%)	12 (85.7%)	11 (78.5%)	12 (85.7%)	10 (71.4%)	10 (71.4%)	14 (100%)
3.	*Acinetobacter* sps n=4	3 (75%)	3 (75%)	4 (100%)	3 (75%)	3 (75%)	2 (50%)	3 (75%)	NA
4.	*Serratia* sps n=2	2 (100%)	1 (50%)	2 (100%)	2 (100%)	2 (100%)	1 (50%)	2 (100%)	NA
5.	*Proteus*sps n= 3	2 (66.6%)	3 (100%)	3 (100%)	2 (66.6%)	3 (100%)	2 (66.6%)	3 (100%)	NA

Note: LE - Levofloxacin, MEM- Meropenem, CAC- Ceftazidime clavunlate, CAZ- Ceftazidime, PIT- Piperacillin tazobactum, AK- Amikacin, TGC- Tigecycline, CL- Colistin, NA- Not Applied

Table 8: Antibiotic Susceptibility Pattern of Gram Positive Cocci

S.NO	ISOLATE	CX	CAZ	CXM	IPM	AZM	VA	CD	AMC	LZ
1.	*Staphylococcus aureus* n=10	5 (50%)	9 (90%)	6 (60%)	8 (80%)	8 (80%)	10 (100%)	7 (70%)	7 (70%)	10 (100%)
S.NO	ISOLATE	CZ	AZM	VA	IPM	AMC	CD	CIP	CXM	
1.	*Streptococcus pneumoniae* n=21	17 (80.9%)	16 (76%)	19 (90.4%)	20 (95.2%)	15 (71.4%)	18 (85.7%)	11 (52.3%)	10 (47.6%)	

Note: CX - Cefoxitin, CAZ- Ceftazidime,CXM- Cefuroxime,IPM- Imepenem, AZM- Azithromycin, VA- Vancomycin, CD- Clindamycin, AMC- Amoxyclav, LZ- Linezolid, CZ- Cefazoline, CIP-Ciprofloxacin

Table 9: Antifungal Susceptibility Of Yeasts

S.NO		AP	CC	FC	ITC	KTC
1.	Candida albicans n=9	8(88.8%)	5(55.5%)	6(66.6%)	6(66.6%)	4(44.4%)
2.	Candida tropicalis n=12	10(83.3%)	7(58.3%)	7(58.3%)	6(50%)	7(58.3%)
3.	Candida krusei n=5	4(80%)	0	3(60%)	3(60%)	3(60%)
4.	Candida glabrata n=4	4(100%)	2(50%)	2(50%)	2(50%)	3(75%)
5.	Cryptococcus n=6	6(100%)	NA	5(83.3%)	4(66.6%)	3(50%)

AP- Amphotericin B ; CC- Clotrimazole ; FC – Fluconazole; ITC – Itraconazole; KTC- Ketoconazole ; NA- Not Applied

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DISCUSSION

It is estimated that about one third of the world's population is infected with MTB. The role of secondary bacterial infection with potentially pathogenic organisms has been hypothesised in patients with prolonged fever after treatment for tuberculosis and in patients who appear to improve clinically "more rapidly" when treated with both anti tuberculosis drugs and standard antimicrobial agents.

Colonisation of potentially pathogenic bacteria is a marker of a patient group with malnutrition and depressed immunity with inability to clear inhaled pathogens. Secondary infection with potentially pathogenic microorganisms is associated with poor prognosis.In the present study most of the samples were from the age groups 31-50 years(53%)with a male to female ratio of 4:1 which correlates with Elizabeth-Nyamvura Mwaura et al[7] and AkitSugu-Furu Moti et al[8], Hayat Khadhumet al [9]and J.Mowna et al[10].

In our study secondary infection was observed in 88% and negative in 12% which correlates with Hayat Khadhum et al[9] who reported 72% and 28%.GNB were 59.3% and the predominat isolate was *Klebsiella* species (48.8%) in the present study which correlates with E.shaddock et al[11] (88.08% and 50%). *Pseudomonas* species were isolated in 31.1% in the present study and SeharAfrhan Nazi et al[2] reported 15.8% in patients with complications and 6.6% in patients with no complications.

Among GPC *Streptococcus pneumoniae* were isolated in 67.7% followed by *Staphylococcus aureus* in 32.3% which correlates with Hayat Khadhum et al[9] who reported 55.6% and11.1%.

In the present study 80% of Enterobacteriaceae were ESBL producers which correlates with E.shaddock et al[11](75%).Among *Staphylococcus aureus* isolates 50% were MRSA strains in the present study. ManayoshiHosodha et al[12] reported an outbreak of MRSA in a TB ward and isolated 17 MRSA isolates from 15 inpatients. Out of 88 culture positive samples 36(40.9%) yeasts were isolated which correlates with Arunavakali et al[3] (40%) and Yahaya .H et al[4] who reported 37%.

Candida albicans was isolated in 25% cases in our study whereas Yahaya.H et al[4] reported 9.3% and Arunavakali et al[3] reported a higher incidence of 50%.*Candida nonalbicans* were isolated in 58.3% in the present study which correlates with Yahaya.H et al[4](64.8%) and Arunavakali et al[3](50%).Among *Candida nonalbicans, Candida tropicalis* was the predominant isolate (33.3%) which correlates with Arunavakali et al[3](20%) , *Candida krusei* (13.8%) correlates with Yahaya.H et al[4](12%) and *Candida glabrata* (11.1%) which correlates with Arunavakali et al[3](20%).

In the present study *Cryptococcus* was isolated in 16.7% where as Elizabeth Nyambura Mwaura et al[9] reported 4.4%.*Candida* species were more sensitive to Amphotericin B followed by Fluconazole ,Itraconazle and Ketoconazole which correlates with Sachin C Deorukhkar et al.[13]*Cryptococcus* species were more sensitive to Amphotericin B which correlates with M.A.Pfaller et al[14].

In the present study 3.45% were MDRTB which correlates with AkitSuguFuru Moto etal[8] (2%).In the present study, 85.7% smear negative patients were co-infected with drug resistant potentially pathogenic microorganisms which may be one of the reasons for persistence of symptoms and misleading the diagnosis of the patients as MDRTB suspects.

CONCLUSION

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1. As there is fast emergence of drug resistance, a constant monitoring of antibiogram of opportunistic flora which act as potentially pathogenic microorganisms is recommended.
2. As the prevalence of *Candida nonalbicans* and *Cryptococcus species* is increased, routine screening for fungal co-infection is needed.
3. In the present study 85.7% of smear negative patients were co-infected with multidrug resistant potentially pathogenic microorganisms which may be one of the reasons for the patients to be diagnosed as MDRTB suspects.

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