ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Evaluation of Antibiotic Supplements for Culturing *Mycobacterium Tuberculosis* from Mouse Macrophages and Organs

Raman Preet Singh, Sarbjit Singh Jhamb*, Prati Pal Singh

Department of Pharmacology and Toxicology, National Institute of Pharmaceutical Education and Research, S. A. S. Nagar – 160 062, Punjab, India

ABSTRACT

Mycobacterium tuberculosis is a slow-growing bacterium and requires long durations of incubation (4-6 weeks) for colonies to appear on solid culture resulting in microbial contamination of culture medium. We investigated the effectiveness of antibiotics in controlling microbial contamination in Middlebrook (7H10 and 7H11) medium and BACTEC 12B vials inoculated with mycobacteria. Incorporation of antibiotics in Middlebrook media and BACTEC 12B medium inhibited microbial contamination in media inoculated with organ homogenates and cell lysates of M. tuberculosis H37Rv-infected mice and macrophages, respectively, without significant effect on mycobacterial growth except with amikacin which slightly suppressed mycobacterial growth as well. Similar results were obtained for log-phase cultures of M. tuberculosis H37Rv, M. tuberculosis H37Ra, M. smegmatis BCG, M. tuberculosis Erdman strain and a multidrug resistant (MDR) M. tuberculosis clinical isolate. In conclusion, addition of antibiotics in Middlebrook and BACTEC 12B medium could reduce microbial contamination in experimental samples without significantly affecting mycobacterial growth.

Keywords: Mycobacterium tuberculosis; microbial contamination; amphotericin-B; amikacin; PANTA.

Corresponding author
E-mail: sarbjitjhamb@rediffmail.com

ISSN: 0975-8585

INTRODUCTION

Tuberculosis (TB) has emerged as a major health burden globally. There are an estimated 2 billion persons who have been exposed to tubercle bacilli, with an estimated 8 million new cases and 2-3 million deaths annually [1]. In such a scenario, it becomes imperative to develop new drugs and efficient methods of diagnosis for control of TB. However, the drug screening and diagnosis is hampered by various problems associated with the tubercle bacilli. First, the bacterium is pathogenic in nature and requires highly specialized laboratories for handling and experimentation. Secondly, mycobacterium is a slow-growing organism and requires long durations of incubation for colonies to appear [2]. Hence, highly nutritive media are used to hasten the growth of mycobacteria resulting in reduction in detection time. However, these highly nutritive media can support the growth of other microorganisms also resulting in high contamination rates [3-7]. It is a common observation that long duration times can result in fungal growth [8]. Further, mycobacterial growth in Petri plates is highly prone to contamination [9]. Although the culture media contain malachite green as an anti-fungal agent (Middlebook 7H10 and Middlebrook 7H11 medium), the medium is unable to control fungal growth. Addition of higher concentrations of malachite green (acid egg medium, IUT medium, Lowenstein-Jenson/LJ medium and Ogawa medium) leads to slower growth and, hence, delayed detection of mycobacteria. Several modifications of conventional media, containing antibacterial compounds, have been reported to reduce the incidence of microbial contamination [7, 9-19]. In recent years, BACTEC method has gained enormous popularity for detection of mycobacterial species due to rapid detection and low contamination rates. The microbial contamination in BACTEC vials can be further reduced by supplementing with PANTA [20]. It may be argued that the contamination may appear because of clinical sampling and inadequate decontamination procedures. However, the fungal contamination can appear in non-clinical, experimental samples also [2, 15]. In the present study, the feasibility of addition of antimicrobial agents in culture medium, to reduce contamination rates without affecting *M. tuberculosis* growth, was investigated.

MATERIALS AND METHODS

Animals

Swiss mice (20 $\pm$ 2g, either sex) were obtained from Central Animal Facility of the Institute, housed in 12 h light/dark cycle and provided food and water *ad libitum*. All studies were approved by Institutional Animal Ethics Committee.

Drugs and culture media

Amphotericin-B (AMP-B), Dulbecco's modified Eagle's medium, fetal bovine serum and Hank's balanced salt solution were purchased from PAA Lab, Austria. Amikacin (AMK), Middlebrook 7H9 medium, Middlebrook 7H10 medium, Middlebrook 7H11 medium and ADC supplement were purchased from HiMedia, India. BACTEC 12B medium and PANTA were purchased from BD Biosciences, USA.

ISSN: 0975-8585

Bacteria and infection

M. tuberculosis H37Rv, *M. tuberculosis* H37Ra, *M. tuberculosis* Erdman strain, a multidrug resistant (MDR) *M. tuberculosis* clinical isolate resistant to first line antitubercular drugs (isoniazid, rifampicin and ethambutol), *M. smegmatis* and BCG were obtained from Tuberculosis Research Centre, Chennai, India. The bacteria were maintained in Middlebrook 7H9 medium supplemented with 10% ADC. Mice were infected intravenously with *M. tuberculosis* H37Rv, sacrificed on day +30 of infection and mycobacterial load in lungs and spleen was assessed as described elsewhere [21]. The organ homogenates were inoculated (50 □ l) on Middlebrook 7H10 or 7H11 medium with or without (control) antibiotics (AMPB or AMK). In parallel, 100 □ l organ homogenates were inoculated in BACTEC vials with or without (control) PANTA. In parallel experiments, organ homogenates of uninfected animals were inoculated on Petri plates or in BACTEC vials in presence or absence of antibiotics or PANTA, respectively.

Intramacrophage killing

Peritoneal macrophages were infected with *M. tuberculosis* H37Rv, lysed on day +7 of infection and mycobacterial load was obtained as described elsewhere [21]. The macrophage lysates were inoculated on Middlebrook 7H10 or 7H11 medium (50 □ l) with or without (control) antibiotics and in BACTEC vials (100 □ l) with or without (control) PANTA. In parallel experiments, macrophage lysates of uninfected macrophages were inoculated on Petri plates or in BACTEC vials in presence or absence of antibiotics or PANTA, respectively.

Antibiotic supplements

Appropriate quantities of AMP-B and AMK were added to ADC supplement. Middlebrook 7H10 or 7H11medium was autoclaved and mixed with AMP-B- or AMK-containing-ADC supplement to obtain a final concentration of 10% v/v ADC. The final concentrations of AMP-B and AMK in medium were 5 and 10 □ g/ml. PANTA supplement was added to BACTEC 12B vials as per manufacturer's guidelines.

RESULTS AND DISCUSSION

M. tuberculosis is a slow-growing bacterium and requires about 3-4 weeks of incubation for colonies to appear on solid medium. Due to the long duration of incubation, the cultures of *M. tuberculosis* are pre-disposed to contamination by microbes, particularly fungi [9]. Though the solid media used for CFU enumeration contain malachite green, but it is ineffective in controlling the fungal contamination [8]. We have observed that the fungi grow at a slow pace as compared to bacteria. The bacterial contamination becomes evident within the first week, while fungal contamination appears in 2nd or 3rd week of incubation. The bacterial colonies tend to remain restricted in a small area whereas the fungal hyphae tend to cover entire surface and can contaminate the entire plate within a week. In our experience, fungal contamination appeared to be a major hindering factor in CFU enumeration as compared to bacterial

contamination as fungal contamination of inoculated plates does not allow estimation of CFUs. We tested the efficacy of antibiotics in controlling microbial contamination and its effect on the growth of *M. tuberculosis*.

We tested the effect of antibiotics on bacterial growth in two commonly employed methods for estimating bacterial load: BACTEC and plating method. The growth of *M. tuberculosis* and microbial contamination on Middlebrook 7H10 and 7H11 was observed in presence and absence of AMP-B and AMK. It was observed that AMP-B did not inhibit the growth of *M. tuberculosis* at 5 and 10 □ g/ml concentrations. However, the fungal contamination was significantly suppressed at the concentrations tested. The results were confirmed for log phase bacterial suspension, organ homogenates and macrophage lysates (Table 1). Further, 5 □ g/ml AMK did not inhibited microbial contamination while 10 □ g/ml significantly inhibited microbial contamination as well as *M. tuberculosis* CFU counts (Table 1). The inhibition of mycobacterial growth in solid medium observed with AMK has also been reported for other antibiotics also [9, 22]. The CFU counts and microbial contamination rates were not significantly different on Middlebrook 7H10 and 7H11 medium (data not shown). Table 1 represents the combined CFU counts and microbial contamination on Middlebrook 7H10 and 7H11 medium. The addition of AMP-B and AMK in Middlebrook 7H10 and 7H11 medium showed similar effects in media inoculated with log-phase cultures of *M. tuberculosis* H37Ra, *M. tuberculosis* Erdman strain, *M. tuberculosis* MDR, *M. smegmatis* and BCG as observed with *M. tuberculosis* H37Rv (data not shown). The log-phase cultures were chosen as bacteria are most susceptible to growth inhibition by antibiotics in the log-phase of growth.

As in case of solid medium, antibiotic supplement (PANTA) resulted in complete suppression of contamination and slight inhibition of mycobacterial growth in BACTEC 12B medium. It was observed that PANTA supplement completely inhibited microbial contamination (assessed by acid-fast staining and Gram-staining) and slightly retarded *M. tuberculosis* growth in PANTA-supplemented BACTEC 12B vials. Addition of PANTA supplement resulted in delayed detection of *M. tuberculosis* by 1-2 days. The results were confirmed for log phase bacterial suspension, organ homogenates and macrophage lysates (Table 2). This is in agreement with an earlier study showing slight suppression of *M. kansasii* growth by PANTA [20]. The addition PANTA in BACTEC 12B medium showed similar results in vials inoculated with log-phase cultures of *M. tuberculosis* H37Ra, *M. tuberculosis* Erdman strain, *M. tuberculosis* MDR, *M. smegmatis* and BCG as observed with *M. tuberculosis* H37Rv (data not shown).

The major source of contamination appeared to be environmental. Several studies reporting the use of clinical specimen have highlighted the role of transportation time in contamination of clinical specimen [4, 23]. However, very few studies have investigated and reported contamination in non-clinical samples. The microbial contamination in non-clinical and uninoculated plates appears due to environmental conditions [9]. We have observed that despite the best possible efforts to control contamination, fungal growth posed a major challenge, particularly in seasons of high humidity and rains. Further, higher contamination rates in organ homogenates as compared to log-phase *M. tuberculosis* cultures and

ISSN: 0975-8585

Table 1

Sample type	Control		AMP-B (5 g/ml)		AMP-B (10 g/ml)		AMK (5 g/ml)		AMK (10 g/ml)	
	CFU counts (%)	Microbial contamination (%)	CFU counts (%)	Microbial contamination (%)	CFU counts (%)	Microbial contamination (%)	CFU counts (%)	Microbial contamination (%)	CFU counts (%)	Microbial contamination (%)
Control (uninoculated)	0	0	0	0	0	0	0	0	0	0
Lung homogenates (uninfected)	0	10	0	5	0	0	0	10	0	5
Spleen homogenates (uninfected)	0	9	0	5	0	0	0	10	0	4
Macrophage lysates (uninfected)	0	0	0	0	0	0	0	0	0	0
Log-phase culture (infected)	100	0	104	0	99	0	100	0	91	0
Lung homogenates (infected)	100	15	98	6	99	1	100	18	79	9
Spleen homogenates (infected)	100	10	96	5	94	0	100	9	81	6
Macrophage lysates (infected)	100	0	99	0	95	0	100	0	85	0

ISSN: 0975-8585

Table 2

Sample type	Control			PANTA		
	GI values (%)	Microbial contamination (%)	Days for detection	GI values (%)	Microbial contamination (%)	Days for detection
Control (uninoculated)	0	0	-	0	0	-
Lung homogenates (uninfected)	0	6	-	0	0	-
Spleen homogenates (uninfected)	0	4	-	0	0	-
Macrophage lysates (uninfected)	0	5	-	0	0	-
Log-phase culture (infected)	100	0	1	101	0	2
Lung homogenates (infected)	100	7	6	99	0	8
Spleen homogenates (infected)	100	5	7	99	0	8
Macrophage lysates (infected)	100	6	5	96	0	6

macrophage lysates indicate that animals could be a possible source of contamination. The possible sources of contamination may include, but not restricted to, hair and skin of mice. We observed that the type of microbes present in hair and skin samples was similar to contaminating microbes (data not shown). The microbes obtained from finger swabs were different from contaminating microbes thus excluding contamination from the user. The surface disinfection with alcohol was able to control bacterial contamination but not fungal contamination, probably because of resistant of fungal spores to alcohol.

CONCLUSION

In conclusion, addition of AMP-B to Middlebrook 7H10 and 7H11 medium and PANTA to BACTEC 12B medium could help in control of microbial contamination without affecting mycobacterial growth in organ homogenates and macrophage lysates.

ACKNOWLEDGEMENT

We are grateful to Director, National Institute of Pharmaceutical Education and Research (NIPER), for his help and encouragement. We thank Mr. Vijay Kumar Misra for excellent technical assistance.

REFERENCES

[1] Dye C. Lancet 2006; 367: 938-940.
[2] Jhamb SS, Singh RP, Singh PP. Ind J Tuber 2008; 55: 70-76.
[3] Cornfield D, Beavis K, Greene J, Bojak M, Bondi J. J Clin Microbiol 1997; 35: 2068-2071.
[4] Leitritz L, Schubert S, Bucherl B, Masch A, Heesemann J, Roggenkamp A. J Clin Microbiol 2001; 39: 3764-3767.
[5] Pfyffer GE, Welscher H-M, Kissling P, Cieslak C, Casal MJ, Gutierrez J, Rusch-Gerdes S. J Clin Microbiol 1997; 35: 364-368.
[6] Tortoli E, Cichero P, Piersimoni C, Simonetti M, Gesu G, Nista D. J Clin Microbiol 1999; 37: 3578-3582.
[7] Alados J, Pareja L, Rosa Mdl. Eur J Clin Microbiol Infect Dis 1998; 17: 731-733.
[8] Schafer M, Fernback J, Ernst M. Aerosol Sci Tech 1999; 30: 161-173.
[9] Mitchison DA, Allen BW, Carrol L, Dickinson JM, Aber VR. J Med Microbiol 1972; 5: 165-175.
[10] Gruft H. J Bacteriol 1965; 90: 829-.
[11] Rothlauf M, Brown G, Blair E. J Clin Microbiol 1981; 13: 76-79.
[12] Yajko D, Wagner C, Tevere V, Kocagoz T, Hadley W, Chambers H. J Clin Microbiol 1995; 33: 1944-1947.
[13] Matajack ML, Bissett ML, Schifferle D, Wood RM. Am J Clin Pathol 1973; 59: 391-397.
[14] McClatchy JK, Waggoner RF, Kanes W, Cernich MS, Bolton TL. Am J Clin Pathol 1976; 65:412-415.

[15] Mitchison DA, Allen BW, Lambert RA. J Clin Pathol 1973; 26: 250-252.
[16] Scarparo CPC, Cichero P, Pezzo MD, Covelli I, Gesu G, Nista D, Scagnelli M, Mandler F. Diagn Microbiol Infect Dis 1999; 34: 293-299.
[17] Whyte T, Cormican M, Hanahoe B, Doran G, Collins T, Corbett-Feeney G. Diagn Microbiol Infect Dis 2000; 38: 123-126.
[18] Mitchison D, Allen B, Manickavasagar D. J Clin Pathol 1983; 36: 1357-1361.
[19] Covert T, Rodgers M, Reyes A, GN Stelma. Appl Environ Microbiol 1999; 65: 2492-2496.
[20] Conville P, Andrews J, Witebsky F. J Clin Microbiol 1995; 33: 2012-2015.
[21] Singh RP, Jhamb SS, Singh PP. Life Sci 2008; 82: 308-314.
[22] Chang CL, Park TS, Oh SH, Kim HH, Lee EY, Son HC, Kim CM. J Clin Microbiol 2002; 40: 3845-3847.
[23] Pinheiro MD, Ribeiro MM. Clin Microbiol Infect 2000; 6: 171-173.