Mycobacterial Diseases
ISSN: 2161-1068

Das et al., Mycobact Dis 2018, 8:2
DOI: 10.4172/2161-1068.1000261

Research Article Open Access

Mitigating PCR /Amplicon Contamination in a High Risk High Burden Mycobacterial Reference Laboratory in a Resource Limited Setting

Prasanta Kumar Das*, Somtirtha B Ganguly and Bodhisatya Mandal

Intermediate Reference Laboratory, State TB Demonstration cum training Centre Kolkata-10 West Bengal, India

***Corresponding author:** Prasanta Kumar Das, Intermediate Reference Laboratory, State TB Demonstration cum training Centre Kolkata-10 West Bengal, India, Tel: 9433355536; E-mail: pkdimmunology@gmail.com

Received date: May 11, 2018; **Accepted date:** May 25, 2018; **Published date:** June 01, 2018

Copyright: © 2018 Das PK, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Abstract

Background: Nucleic acid amplification techniques have become important machineries in the diagnosis of several diseases in clinical laboratories. PCR contamination/Amplicon Contamination leading to false positivity remains a major concern in these laboratories. Prevention of these contaminations in establishing these Molecular Biology Laboratories has been very crucial over the years. Though closed system PCRs has substantial reduction in the PCR contamination rates the conventional probe based hybridization methods continues to show occurrence of contamination for various reasons.

The Study involved checking the crucial parameters as well as the probable candidates of causing the contamination at a high burden setting. Bringing out the most effective interventions in controlling PCR contaminations for future endeavors stood as a priority. The study explored the efficacies of different sets of interventions contributed in the process of reducing the contaminants.

Materials and Methods: The detection of the contaminating PCR products or amplicons or contaminating organism is done by the Genotype MTBDR plus V2 kits (Hains Life Sciences) based on DNA strip technology

Results: The pre and post cleaning as well as cleaning of the working surfaces was able to bring down the mean contamination percentage by 36.5%. The combined effect of the cleaning of the work surfaces, the automated pipetting devices and the AC machines along with it filters were able bring down the mean contamination percentage to 53.5% reducing the rate contamination nearly to between 94.6% (mean percentage contamination was 56.5% at the control run).

Keywords: PCR contamination; Amplicon contamination; *Mycobacterium tuberculosis* Complex (MTBC)

Introduction

Amplicon contamination when encountered is the most dreaded experience for any molecular biology laboratory. The public health TB laboratories at the intermediate level has probe based molecular detection test that requires strategies to avoid PCR contamination. Amplified PCR products as contaminants giving false positives may affect the patient care severely [1,2]. Validity of an entire batch based on its detection with oligonucleotide probes is at question when the negative control shows presence of contaminating amplicons incurring waste of resources.

The Intermediate Reference laboratory at Kolkata under State TB demonstration and training centre (Govt. of West Bengal India) has a molecular biology setup that runs Line probe assay for anti-tubercular drugs towards first line (isoniazid and rifampicin) and second line (fluoroquinolones and Second line injectables) drugs. The laboratory is well managed with Internal QC and External QC done by the National Reference laboratories on behalf of Central TB Division, Govt. of India.

The Laboratory encountered an occurrence of Amplicon contamination that recurred in subsequent runs (January 2016). The Laboratory involves a process of lysis buffer based DNA extraction followed by PCR and hybridizing with biotinylated oligo nucleotide probes on nitrocellulose strips. When a Polymerase Chain Reaction is in progress more than 10 million copies of a template DNA are produced [3]. The aerosolized amplified product may bind to the oligonucleotide probe resulting a false positivity.

These loose amplified nucleic acid moieties are termed as Amplicons which may eventually contaminate the environment. IRL Kolkata is a high risk high burden laboratory that involves 80 to 100 sample processing load daily on an average. About 24 DNA extracts reaches the Molecular Biology set up after initial processing. This means a billion of copies of amplified PCR products are generated on regular basis. The huge quantum of this amplified PCR products are hence to be decontaminated regularly in order to avoid false positives. The PCR machine, workstations, micropipettes and automated pipetting devices are cleaned and calibrated for ensuring valid results. The man power involved was trained by trainers from National reference laboratories and had a clear understanding of the amplicon control mechanisms.

The Handling with proper maneuvers and commendable dexterity was ascertained. Despite of these measures contamination occurred as it was evident from the control runs. The negative control showed presence of bands. Subsequent exercise involved conduction of onsite evaluation and addressing the shortfalls based on the experiences and

observations of various studies [4-8]. The contamination persisted even after addressing the base line short falls. Each episode of sentinel testing preceded after a thorough cleaning procedure based on the Molecular Detection of drug resistant tuberculosis by line probe assay [9]. While mitigating amplicon contamination at IRL Kolkata the following objectives were kept in consideration.

- To establish that, a significant contamination has occurred.
- To find out the source of amplicon contamination.
- To find out the Most contaminated room.
- To find out the area of the facility maximally affected by the amplicon contamination and its possible causes.
- The most significant interventions in controlling the contamination.
- To provide recommendations based on the inference obtained.

Materials and Methods

Basic compliances and internal quality checks for contamination control are in place. The entire facility design has three distinct separate units. The master mix section, the amplification or PCR unit and the detection unit. The Master Mix unit involves preparation of reagents with salts, primers and Taq polymerase. The Amplification unit ensures the actual polymerase chain reaction. The detection unit involves hybridization with the oligo nucleotide probes in order to detect the amplified genetic sequences. The MTBDR V2 kits of Hains life sciences for the detection of MTBC PCR products were used.

As the lab was operating only one the first line of drugs the MTBDR V2 kit for the detection of first line of drugs, this kit were taken to detect for the amplicons that are produced during PCR of the gene segments primarily responsible for resistance to the first line of drugs.

The different areas to be checked for the amplicon contaminations were placed with Cryo vials containing DNase, RNase free sterile molecular water. This will help in capturing the airborne amplicons as a comparable tool test for the paired data and checking on the minimal standard deviations of the mean in order to establish a significant contamination at a particular site another set of DNase RNase free Lysis Buffer were taken and placed in the same area to check on the air borne amplicons.

The molecular grade water as well as the Lysis buffer (sterile DNase RNase free) was then put for DNA extraction and subsequently amplication was done. Contaminating known set of amplicons whose primers are already present in the Master Mix would get amplified indicating contaminants in that particular site. Both the Molecular grade water and the lysis buffer were left open to catch up the air borne amplicons analogous to that of the settle plate technique save for the part that this is purely a molecular detection.

Testing for contaminants involved DNA extraction by lysis buffer and subjecting them for PCR. Detection of the segments was done by using kit based oligonucleotide probes on nitrocellulose paper strips. The PCR contaminants within the following gene segments were tested for (Figure 1).

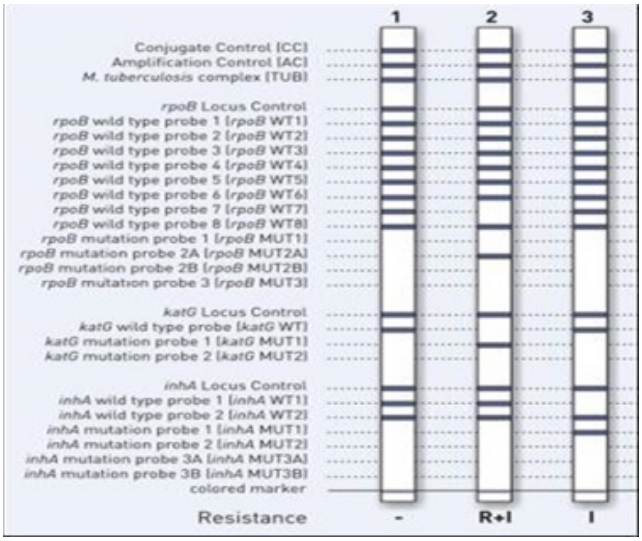

Figure 1: Tested for the amplicon.

Study design

Mechanical barriers have proved to be very effective in controlling carry over contamination. Separate zones dividing separate areas of operation depending on the status of their cleanness provides significant control over the spread of the amplicons [10].

Based on the above stated conditional requisites the molecular biology set up at the Intermediate reference laboratory Kolkata includes three separate clean rooms for designated activities. The Master Mix room (MMX) where the PCR reagents are prepared with the desired primers nucleotides buffer and Taq polymerase. The amplification section where PCR was performed after addition of the extracted DNA (AMP) the detection room where the PCR products are exposed to the nitrocellulose strips containing oligonucleotide probes and finally detected by a color development over the strips (DET).

23 sites are selected in each of the three sections namely Master mix section, Amplification section and detection section. A total of three runs in two batches were performed in these sites. The first batch comprised of Molecular grade water (MGW) and the second batch comprised of lysis buffer (LB). The Cryo vials containing molecular grade water or lysis buffer were allowed to stand at those sites overnight in order to capture the amplicons. Significant contamination is ascertained by calculating odds ratio between the chances of occurring a contamination over not occurring a contamination in all the rooms. The population (Confidence interval) was calculated taking the 414 runs (sample size 414) the frequency was taken as the numbers of occurrence of contamination i.e. 133. The p value at 95% confidence limit was between 0.27 to 0.36.

As the source of contamination being airborne, the figuring out of the exact source becomes difficult. Sequentially ruling out each and every step, meticulously performing a specific intervention and checking the status of contamination one after the other might provide some evidence based insights but being conclusive would require a significant decrease in contamination by a particular intervention at a particular site in subsequent tests. Contamination site that showed

recurrent contaminations even after repeating the tests after all the intervention may suggest a site in close proximity qualifying for a source suspect.

Each section is tested for a mean percentage contamination. This is calculated out for the three runs for all the 23 sites. A mean percentage for each room is detected. The room or section showing the maximum mean percentage contamination is taken as the most contaminated room or section.

It is always not likely that the room or section with the maximum mean percentage contamination would harbor the source of contamination but a substantial chance is an obvious phenomenon. The site that scores higher percentage contamination from the mean percentage contamination with maximum variance on higher side considering all the three runs were taken as the site that is maximally contamination. Onsite evaluation and introducing suitable interventions would further help in finding out the cause.

The study explores twelve interventions and their effects in minimizing the mean percentage contaminations of each section.

- Facility design
- Work flow
- PPE adherence and controlled skillful dexterity
- Proper disposal care and concerns
- Proper preparation of cleaning reagents and disinfectants
- Cleaning of floors
- Cleaning of walls
- Cleaning of work surfaces (prior and Post operations)
- Cleaning of pipettes (prior and post operations)
- Cleaning of automated pipetting device/PCR machine
- Use of Ultraviolet radiations (intensity and effective exposure)
- Cleaning of air conditioners

The effect on mean percentage contamination in all the three rooms by each of this individual is tested. Table 1.3 showing the intervention with corresponding decrease in the mean percentage contamination can serve as an illustrated explicit depiction. The intervention showing maximum decrease in mean percentage concentration in all the three rooms will be assigned as the most effective intervention in controlling amplicon contamination.

Results

A sample size of 414; 23 (sites) x 3 (runs) x 2 (first with MGW and then with LB) x 3 (for three rooms) a frequency is that occurrence of contamination in 414 runs was 133 (absolute figures out of 414). A population confidence interval of 95% with 414 as a sample size and 133 as a frequency showed a confidence interval proportion ranging from 0.27 to 0.36. The mean contamination percentage was found to be highest in the detection room. However it was observed effective contamination control in the Master Mix and amplification room would minimize the chances of contamination in the detection room (Table 1.1).

Contamination			Non Contamination	Odds ratio
MMX	MGW	21	48	2.91
	LB	9	60	
AMP	MGW	31	38	2.71
	LB	16	53	
DET	MGW	44	25	10.4
	LB	10	59	

Table 1.1: Odds ratio of the three rooms.

As seen from the odds ratio the chances of occurring an event of contamination is higher than an event of non-contamination but the chance in case of the detection room is high as compared to the MMX and AMP section. The overall mean percentage contamination of the MMX, AMP and DET was found to be 31%, 34% and 39.1%. The detection room shows maximum contamination of mean percentage contamination of 39.1% (Table 1.2).

		MGW	LB	Over all
Mean Percentage Contamination	MMX	30.40	13.04	31.00
	AMP	42.02	26.08	34.00
	DET	63.70	14.40	39.1

Table 1.2: The mean percentage contamination in the three rooms

The effects of the intervention in decreasing the mean contamination percentage of the rooms showed significant reduction in contamination rates by cleaning activities. Mechanical barriers have proved to be very effective in controlling carry over contamination. Separate zones dividing separate areas of operation depending on the status of their cleanness provides significant control over the spread of the amplicons. A unidirectional flow is maintained from the reagent preparation area to the sample preparation area, the amplification area, and finally to the detection area. The chemical decontamination has been observed to be the most effective of all mechanisms. All the equipment such as pipetting devices, Thermo cycler, Genotype blotting machines, mini spin were cleaned with 1% hypochlorite solution (NaOCl) prior and post operation. As stringent and aggressive intervention to overcome the PCR contaminant load 4% Sodium hypochlorite was used [10].

Bleach helps in causing oxidative damage to nucleic acid [11]. Each episode of cleaning with hypochlorite followed by cleaning the surfaces with 70% ethanol in order to nullify the corroding effects of bleach [10]. The pre and post cleaning as well as cleaning of the working surfaces was able to bring down the mean contamination percentage by 36.5%. The combined effect of the cleaning of the work surfaces, the automated pipetting devices and the AC machines along with it filters were able bring down the mean contamination percentage to 53.5% reducing the rate contamination nearly to between 94.6% (mean percentage contamination was 56.5% at the control run used in the three rooms (Table 1.3 and Table 1.4).

Sr. No.	Decrease in mean percentage contamination from 56.5%			
	Interventions			
1.	Facility design	25%	35.50%	53.50%
2.	Work flow	25%	35.50%	53.50%
3.	PPE adherence and controlled dexterity	25%	35.50%	53.50%
4.	Proper disposal and other aseptic measures	25%	35.50%	53.50%
5.	Proper preparation Of cleaning reagents and disinfections		35.50%	53.50%
6.	Cleaning floors		35.50%	53.50%
7.	Cleaning of walls		35.50%	53.50%
8.	Cleaning of work surfaces (prior and post operations)		35.50%	53.50%
9.	Cleaning of pipettes (Prior and post operations)		35.50%	53.50%
10.	Cleaning of Automated Pipetted device (GT blot)/PCR machine			53.50%
11.	Exposure of ultra violet radiations (intensity and exposure time.)			53.50%
12.	Cleaning of the air conditioners			53.50%

Table 1.3: Intervention and their contribution in controlling the mean percentage contamination.

Interventions	Contribution in reducing the contamination by (occurrence of reduction of contamination in absolute number) *39 is the occurrence of contamination in controlled run
Sr. No. 1-4	43.5% (from 39 to 22=17)
Sr. No. 1-9	64.1% (from 39 to 14=25)
Sr. No. 1-12	94.8% (from 39 to 2=37)

Table 1.4: Showing contribution in reducing the contamination by the sets of intervention (annexed in Table 1.3).

Discussion

The Intermediate Reference laboratory at Kolkata is under the State TB demonstration and Training center Govt. of West Bengal. This is an apex institution conducting tests for presumptive MDR and XDR TB cases catering all over the state West Bengal, India. The Laboratory has a molecular biology set up that runs Line probe Assay. During January 2016 the laboratory encountered amplicons contamination in subsequent runs that led to a systemic step by step analysis of the source and most effective intervention in minimizing the contamination. Through a series of sentinel testing after a specific set of interventions the contaminants started decreasing till a stage was reached where three consecutive runs showed no contamination at all.

The exercise helped in inferring some major recommendation and a singular observation that may help the labs performing open PCR systems. The facility is made with puff panels and epoxy flooring for ensuring smooth surfaces facilitating the cleaning process avoiding creation of any niche for accumulation of particulates. The flow of work is ensured in a sequential manner from master mix room to detection room in order to avoid carryover contaminants from one section to other. Reagents being prepared in a clean separate master mix unit prior to the actual polymerization at the PCR unit. This provided additional contamination control measures. Rigorous cleaning of the units before and after operation was ensured with 1% sodium hypochlorite (NaOCl) solution followed by 70% alcohol.

Hypochlorite helped in denaturing the spilled PCR products. 70% alcohol helped in the protein denaturation process towards any other contaminating organisms. Twenty three sites from each of the rooms were selected and the cryo vials containing molecular grade water or lysis buffer were allowed to stand at those sites overnight in order to capture the amplicons. The Odds ratio calculated out between the chances of occurring and not occurring a contamination showed three major findings. It was established that a significant contamination has occurred in all the three rooms.

In all the three rooms the occurrence of contamination when tested with Molecular grade water was higher than the chances of contamination with lysis buffer. As the three runs of the lysis buffer were tested after specific interventions before each runs. The higher chances of contamination occurring with the molecular grade water showed the effectiveness of the interventions in minimizing the contamination rates. The decrease in the mean contamination percentage in these rooms after the execution of these interventions also corroborated to the fact that the interventions played effective roles in combating the contaminants. It was observed that the cleaning of the work surfaces, the automated pipetting devices, PCR machine and the AC machines along with it filters with 1% hypochlorite followed by 70% alcohol and exposure of UV rays significantly lowers down the mean contamination percentage. The UV irradiation sterilizes the PCR contaminants and prevents from amplification [12,13]. Daily cleaning of the UV lights prior and post operations

helped in exposure of the desired intensity. The UV was exposed to the work surface for 20 minutes. Wiping the surfaces with 1 to 10% hypochlorite has been a very effective tool [14]. The cleaning of the working surfaces was able to bring down the contamination rate by 60%. The combined effect of the cleaning of the work surfaces, the automated pipetting devices and the AC machines along with it filters were able bring down the contamination rates between 90 to 95%.

As per the odds ratio calculation, the chances of contamination are 2.91 times more as compared to non-contamination in the Master Mix room (MMX). The chances of contamination are 2.71 times more as compared to non-contamination in the AMP room. The chance is maximum in the detection room this has led us to infer in our study that if contamination control is effectively done in the master mix and amplification rooms, contamination to occur in the detection room can be avoided as the contaminants of the erstwhile rooms cumulatively adds up in the detection room due to the unidirectional work flow.

Conclusion and Recommendations

The Study showed us the effectiveness of cleaning in controlling PCR contamination in high burden high risk laboratory. Regular cleaning the work surfaces, the automated pipetting devices, PCR machine and the AC machines along with it filters with 1% hypochlorite followed by 70% alcohol and exposure of UV rays significantly lowers down the mean contamination percentage. Room wise installation of UV lights apart from the PCR hood, providing at least 125 $\mu W/cm^2$ within one meter of the thermocycler is recommended but care must be taken that the DNA extracts and PCR products are not exposed to it as UV irradiation may denature the enzyme Taq polymerase and oligonucleotide [15]. The intensity of the UV lights must be checked after extensive hours of its use. For G30T8 UV lamp it has been seen the UV output becomes 80% of that a new lamp after 8000 hours of use [16].

As there are a lot of low molecular weight moieties that floats in the vicinity instead of settling down on floors or on the work surfaces UV radiation can effectively control these contaminants. An exposure of 20 minutes with a desired intensity of prior to the operation will be a very effective tool in avoiding PCR contamination. It was found that figuring out the exact source of this air borne PCR contamination is very difficult. The contamination might have occurred during reagent preparation or addition of samples, at any step during sample maneuvers after polymerase chain reaction. The detection room shows highest contamination rate of mean percentage contamination of 39.1% as the sample and reagents flows from MMX to AMP followed by DET.

All the contaminants acquired in MMX and AMP reaches DET to show a maximum contamination. The odds ratio calculation also led to an singular observation reiterating the fact that cleaning of the Master Mix room and amplification room plays a pivotal role in the control of the contamination in the detection room If the contamination control is effectively done in the Master Mix and amplification room the chances of contamination to occur in the detection room is significantly reduced. The study showed us absolute contamination control is hardly a reality after repeated sentinel testing that preceded after rigorous cleaning activites there remained 5 to 10% chances of contamination to occur as this was observed earlier.

"It should be emphasized that despite these improvements, PCR and other amplification techniques remain susceptible to carry-over contamination. False-positive findings have been reported with all the commercially- available automated systems" [10].

Thus it is essential to ensure clean runs in at least two negative controls (one during the preparation of reagents the other during extraction) to validate a batch of probe runs.

Acknowledgement

We are thankful to Dr. Dheeraj Tumu WHO Consultant, RNTCP West Bengal, India for providing statistical inputs and all patients suffering and staffs contributing for their dedication.

References

1. Molloy PJ, Persing DH, Berardi VP (2001) False-positive results of PCR testing for lyme disease. Clin Infec Dis 33: 413-414.
2. Patel R, Grogg KL, Edwards WD, Wright AJ, Schwenk NM (2000) Death from inappropriate therapy for lyme disease. Clin Infect Dis 31: 1107-1109.
3. John Brunstein (2018) Strategies for avoiding amplicon contamination in the Molecular biology laboratory. The Primer.
4. Hartley J, Rashtchian A (1993) Dealing with contamination: Enzymatic control of carryover contamination in PCR. PCR Methods Appl 3: S10-S14.
5. Prince AM, Andrus L (1992) PCR: How to kill unwanted DNA. Biotechniques 12: 358-360.
6. Ou CY, Moore JL, Schochetman G (1991) Use of UV irradiation to reduce false positivity in the polymerase chain reaction. Biotechniques 10: 442-446.
7. Van Pelt-Verkuil E, Belkum AV, Hays JP (2008) Principles and technical aspects of PCR amplification (1st Edn) Springer Netherlands.
8. Viljoen GJ, Nel LH, Crowther JR (2005) Molecular diagnostic PCR handbook (1st Edn) Springer Netherlands.
9. Barnard M, Parsons L, Miotto P, Cirillo D, Feldmann K, et al. (2012) Molecular detection of drug resistant tuberculosis by Line probe Assay. Laboratory manual for resource limited settings. FIND, Geneva, Switzerland pp: 274.
10. Aslanzadeh J (2004) Preventing PCR amplification carry over contamination in a clinical laboratory. Ann Clin Lab Sci 34: 389-396.
11. Hayatsu H, Pan SK, Ukita T (1971) Reaction of sodium hypochlorite with nucleic acids and their constituents. Chem Pharm Bull (Tokyo) 19: 2189-2192.
12. Dwyer DE, Saksena N (1992) Failure of ultra-violet irradiation and autoclaving to eliminate PCR contamination. Mol Cell Probes 6: 87-88.
13. Fox JC, Ait-Khalid M, Webster A, Emery VC (1991) Eliminating PCR contamination: Is UV irradiation the answer? J Virol Meth 33: 375-382.
14. Persing DH (1993) Amplification product inactivation. In: Diagnostic molecular microbiology principles and applications. ASM Press, Washington, DC pp: 105-121.
15. Pao CC, Hor JJ, Tsai PL, Horng MY (1993) Inhibition of in vitro enzymatic amplification reaction by ultra- violet light irradiation. Mol Cell Probes 7: 217-219.
16. Harrington BJ, Valigosky M (2007) Monitoring ultra violet lamps in biological safety cabinets with cultures of standard bacterial strain on TSA blood agar. Lab Med 38: 165-168.