Abstract

Background: Nucleic acid amplification techniques have become important machineries in the diagnosis of several diseases in clinical laboratories. Polymerase chain reaction (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. Although closed system PCRs have substantial reduction in the PCR contamination rates, the conventional probe-based hybridization methods continue 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 the PCR contaminations for future endeavors stood as priority. The study explored the efficacies of different sets of interventions contributed to the process of reducing the contaminants. 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 cleaning the work surfaces, the automated pipetting devices, and the AC machines was able to bring down the mean contamination percentage to 53.5% reducing the contamination rate nearly to 94.6%. Conclusion: Regularly cleaning the work surfaces, the automated pipetting devices, the PCR machine, and the AC machines along with it filters and exposure of UV rays significantly lowers down the mean contamination percentage.

Keywords: Amplicon contamination, Mycobacterium tuberculosis complex, polymerase chain reaction contamination

INTRODUCTION

Amplicon contamination when encountered is the most dreaded experience for any molecular biology laboratory. The public health tuberculosis (TB) laboratories at the intermediate level has probe-based molecular detection test that requires strategies to avoid polymerase chain reaction (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 the presence of contaminating amplicons incurring waste of resources.

The Intermediate Reference Laboratory (IRL) at Kolkata under State TB Demonstration and Training Centre (Government of West Bengal, India) has a molecular biology setup that runs line probe assay for antitubercular drugs toward the first-line (isoniazid and rifampicin) and second-line (fluoroquinolones and second-line injectables) drugs. The laboratory is well managed with internal quality check (QC) and external QC done by the National Reference Laboratories on behalf of Central TB Division, Government 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 (LB)-based DNA
While mitigating amplicon contamination at IRL Kolkata, the following objectives were kept in consideration.

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

Basic compliances and internal QC's 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 detection unit involves preparation of reagents with salts, primers, and Taq polymerase. The amplification unit ensures the actual PCR. The detection unit involves hybridization with the oligonucleotide probes to detect the amplified genetic sequences. The MTBDR V2 kits of Hains Life Sciences for the detection of Mycobacterium tuberculosis complex PCR products were used.

As the laboratory was operating only one the first line of drugs, the MTBDR V2 kit for the detection of first line of drugs, this kit was 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 cryovials containing DNase, RNase-free sterile molecular water. This will help in capturing the airborne amplicons. As a comparable tool test on a paired data and there after checking on the minimal standard deviations from the mean in order to establish significant contamination at a particular site, another set of DNase RNase-free LB (Lysis Buffer) were taken and placed in the same area.

The molecular grade water (MGW) as well as the LB (sterile DNase RNase free) was then put for DNA extraction, and subsequently, amplification 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 MGW and the LB were left open to catch up the airborne 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 LB and subjecting them for PCR. Detection of the segments was done using kit-based oligonucleotide probes on nitrocellulose paper strips. The PCR contaminations within the following gene segments were tested for [Figure 1].

**Methods**

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 provide significant control over the spread of the amplicons. Based on the above-stated conditional requisites, the molecular biology setup at the IRL 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

**Figure 1:** Tested for the amplicon
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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).

Twenty-three 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 MGW and the second batch comprised LB. The cryovials containing MGW or LB were allowed to stand at those sites overnight 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 and 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 proximity to 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 scores higher percentage contamination from the mean percentage contamination with maximum variance on the higher side considering all the three runs were taken from the site that is maximally contaminated. Onsite evaluation and introducing suitable interventions would further help in finding out the cause.

The study explores 12 interventions and their effects in minimizing the mean percentage contaminations of each section.

- Facility design
- Workflow
- Personal Protective Equipment 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 (before and after operations)
- Cleaning of pipettes (before and after operations)
- Cleaning of automated pipetting device/PCR machine
- Use of ultraviolet (UV) 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 shows that:

| Interventions                                      | 1st Set of Run | 2nd Set of Run | 3rd Set of Run |
|---------------------------------------------------|----------------|----------------|----------------|
| Facility design                                   | 25%            | 35.50%         | 53.50%         |
| Workflow                                          | 25%            | 35.50%         | 53.50%         |
| Personal Protective Equipment adherence and controlled dexterity | 25%            | 35.50%         | 53.50%         |
| Proper disposal and other aseptic measures         | 25%            | 35.50%         | 53.50%         |
| Proper preparation of cleaning reagents and disinfections | 35.50%         | 53.50%         | 53.50%         |
| Cleaning floors                                   | 35.50%         | 53.50%         | 53.50%         |
| Cleaning of walls                                 | 35.50%         | 53.50%         | 53.50%         |
| Cleaning of work surfaces (before and after operations) | 35.50%         | 53.50%         | 53.50%         |
| Cleaning of pipettes (before and after operations) | 35.50%         | 53.50%         | 53.50%         |
| Cleaning of automated pipetting device/PCR machine | 53.50%         |                |                |
| Exposure of ultraviolet radiations (intensity and exposure time) | 53.50%         |                |                |
| Cleaning of the air-conditioners                  | 53.50%         |                |                |

$^{1}$PCR: Polymerase chain reaction, GT: Genotype, PPE: Personal Protective equipment

Results

A sample size of 414; 23 (sites) $\times$ 3 (runs) $\times$ 2 (first with MGW and then with LB) $\times$ 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 DET. However, it was observed effective contamination control in the MMX and AMP would minimize the chances of contamination in the DET [Table 2].

As seen from the odds ratio, the chances of occurring an event of contamination are higher than an event of noncontamination, but the chance in case of the DET is high as compared to the MMX and AMP. The overall mean percentage contamination of the MMX, AMP, and DET was found to be 31%, 34%, and 39.1%, respectively. The DET shows maximum contamination of mean percentage contamination of 39.1% [Table 3].

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 cleanliness provides significant control over the spread of the amplicons. Unidirectional flow is maintained from the reagent preparation area to the sample preparation area, the amplification area, and finally the detection area. The chemical decontamination has been observed to be the most effective of all mechanisms. All the equipment such as pipetting devices, thermocycler, genotype blotting machines, and mini spin were cleaned with 1% hypochlorite solution (NaOCl) before and after operation. As stringent and aggressive intervention to overcome the PCR contaminant load, 4% NaOCl was used. [10]

Bleach helps in causing oxidative damage to nucleic acid. [11] Each episode of cleaning with hypochlorite was followed by cleaning the surfaces with 70% ethanol 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 cleaning the work surfaces, the automated pipetting devices, and the AC machines was able to bring down the mean contamination percentage to 53.5% reducing the contamination rate nearly to 94.6% [Table 4].

**Discussion**

The IRL at Kolkata is under the State TB Demonstration and Training Center, Government of West Bengal. This is an apex institution conducting tests for presumptive multidrug-resistant and extremely drug-resistant TB cases catering all over the state West Bengal, India. The laboratory has a molecular biology setup 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 laboratories 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 MMX to detection room to avoid carry-over contaminants from one section to other. Reagents are prepared in a clean separate MMX before 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% NaOCl solution followed by 70% alcohol.

Hypochlorite helped in denaturing the spilled PCR products. Seventy percent alcohol helped in the protein denaturation process toward any other contaminating organisms. Twenty-three sites from each of the rooms were selected and the cryovials containing MGW or LB were allowed to stand at those sites overnight to capture the amplicons. The odds ratio calculated out between the chances of occurring and not occurring contamination showed three major findings. It was established that significant contamination has occurred in all the three rooms.

In all the three rooms, the occurrence of contamination when tested with MGW was higher than the chances of contamination.

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**Table 2: Odds ratio of the three rooms**

| Rooms   | Contaminations | Non Contaminations | ODDS ratio |
|---------|----------------|--------------------|------------|
| MMX     | 21             | 48                 | 2.91       |
| MGW     | 9              | 60                 |            |
| AMP     | 31             | 38                 | 2.71       |
| LB      | 16             | 53                 |            |
| DET     | 44             | 25                 | 10.4       |
| LB      | 10             | 59                 |            |

MMX: Master Mix room, MGW: Molecular grade water, LB: Lysis buffer, AMP: Amplification room, DET: Detection room, OR: Odds ratio

**Table 3: The mean percentage contamination in the three rooms**

| Mean percentage contamination | MGW   | LB    | Over all |
|-------------------------------|-------|-------|----------|
| MMX                           | 30.40 | 13.04 | 31.00    |
| AMP                           | 42.02 | 26.08 | 34.00    |
| DET                           | 63.70 | 14.40 | 39.1     |

MMX: Master Mix room, MGW: Molecular grade water, LB: Lysis buffer, AMP: Amplification room, DET: Detection room

**Table 4: Contribution in reducing the contamination by the sets of intervention (annexed in Table 1)**

| Interventions | Contribution in reducing the contamination by the specific series of intervention |
|---------------|---------------------------------------------------------------------------------|
| The Serial number 1-4 | 43.5% (from 39 to 22=17) |
| Serial number 1-9 | 64.1% (from 39 to 14=25) |
| Serial number 1-12 | 94.8% (from 39 to 2=37) |
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devices, the 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 to and after operations helped in exposure of the desired intensity. The UV was exposed to the work surface for 20 min. Wiping the surfaces with 1–10% 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 to bring down the contamination rates between 90% and 95%.

As per the odds ratio calculation, the chances of contamination are 2.91 times more as compared to noncontamination in the MMX. The chances of contamination are 2.71 times more as compared to noncontamination in the AMP. The chance is maximum in the DET; this has led us to infer in our study that if contamination control is effectively done in the MMX and AMP, contamination to occur in the DET can be avoided as the contaminants of the erstwhile rooms cumulatively add up in the DET due to the unidirectional workflow.

**Conclusion and Recommendations**

In recent times, several relevant works have been done based on line probe assay which involves molecular detection by PCR. 3.90% of mono rifampicin resistance has been shown in a study based on molecular detection. The wide variation among the strains of *M. tuberculosis* isolated from TB cases was reported.16 Treatment-associated death from erstwhile studies implicated the need of molecular-based rapid diagnosis.17 Attempts have been made to probe into the genetic characterization of the gene segments used in line probe assay to mark a specific outcome based on geographic or demographic differences.18

All the aforesaid studies were based on PCR-based studies; thus, the relevance of controlling PCR contamination is of utmost necessity. The reference laboratories would benefit by implementing strict and specific operating and cleaning procedures.

The study showed us the effectiveness of cleaning in controlling PCR contamination in high-burden high-risk laboratory. Regularly cleaning the work surfaces, the automated pipetting devices, the 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 μW/cm within 1 m 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.19 The intensity of the UV lights must be checked after extensive hours of its use. For G30T8 UV lamp, it has been seen that the UV output becomes 80% of that a new lamp after 8000 h of use.20

As there are a lot of low molecular weight moieties that float 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 min with a desired intensity of before the operation will be a very effective tool in avoiding PCR contamination. It was found that figuring out the exact source of this airborne PCR contamination is very difficult. The contamination might have occurred during reagent preparation or addition of samples, at any step during sample maneuvers after PCR. The DET shows the highest contamination rate of mean percentage contamination of 39.1% as the sample and reagents flow 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 a singular observation reiterating the fact that cleaning of the MMX and AMP plays a pivotal role in the control of the contamination in the DET; if the contamination control is effectively done in the MMX and AMP, the chances of contamination to occur in the detection room are significantly reduced. The study showed us that absolute contamination control is hardly a reality after repeated sentinel testing that preceded after rigorous cleaning activities; there remained 5–10% 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.

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**Conflicts of interest**

There are no conflicts of interest.
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