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Reliable Application of PCR: An Elusive Pathway

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The emergence and the wide spread of molecular biology, has undoubtedly transformed the way we think about fundamental and applied research on infectious diseases. Since its invention in the late eighties [1], the impact specifically of the polymerase chain reaction (PCR) on the progress that has been recorded in this field, can be characterized as nothing less than remarkable. Eventually PCR has become a valuable asset for research, and a powerful diagnostic tool especially with connection to the detection of microbial pathogens and genetic variations associated with disease. Today there are an overwhelming number of reports on PCR detection assays about practically every possible diagnostic indicator. Many of these publications refer to the comparative evaluation of selected methodologies whereas in several cases the researchers address issues related to their reliable application. Unfortunately the latter is a subject the significance and the complexity of which is often underestimated when designing an experimental plan. PCR performed within the context of diagnostic or epidemiology studies has to be applied with respect to the technical specifications of the assay that has been selected, and with absolute consideration of how these may affect the results that will be recorded and their interpretation. However, this assessment involves a large number of interacting factors [2], which renders the thorough evaluation of published PCR methodologies a definitely demanding prerequisite for their reliable application. Though it is not the purpose of this article to provide an analysis on this topic, it would be perhaps useful if some points of critical significance were outlined.

Minimum Detection Limit

Minimum detection limit (MDL) i.e., the lowest concentration level which can give a positive test result that can be determined to be statistically different from the blank, at 99% level of confidence [3]. MDL is a property often confused with sensitivity even in articles published in peer review journals, maybe because it is also referred to as analytical, in contrast to clinical, sensitivity. Though MDL is a defining characteristic of the latter, the distinction between the two should be clear since the information that they provide is by no means identical. In this respect the use of the term minimum detection limit should perhaps be considered more appropriate.

The procedure of calculating the MDL of a PCR assay usually involves the preparation of duplicate or triplicate serial dilutions of the targeted analyte. This assessment however has to be designed with respect to the investigation that the specific assay will be used for. Considering that the ability of PCR to amplify a nucleotide sequence in a solution will be affected by the amount and the nature of the "debris" DNA found in it [4], the serial dilutions that will be used to assess MDL should be prepared so that they are representative of the samples that will be tested. Therefore if we aim to apply PCR to the detection of a pathogen in clinical samples, measurement of MDL should be performed on serial dilutions of the same type of sample rather than the pathogen itself. Using dilutions of negative samples spiked with a previously defined amount of the targeted pathogen is an alternative that is often used for practical reasons, since accurately calibrated samples from naturally infected individuals cannot be always made available. This however introduces again a factor of inconsistency specifically with connection to intracellular pathogens. The MDL that will be calculated using the model mentioned above can be significantly different from that of the method applied to clinical samples, since in this case it will depend on the ability of the DNA extraction methodology to release the targeted sequence from within the cell, into the solution that will be used for PCR. For this purpose it would be worth to consider constructing the starting solution of the serial dilutions using cell cultures experimentally exposed to the specific pathogen, which can be considered an adequately accurate model of "wild-type" samples.

Sensitivity

Sensitivity is evaluated by calculating the proportion of test positives which are correctly identified within a population of true positives [5]. Knowing the MDL of our assay and the distribution of the targeted diagnostic marker in the body, we can select the type and the number of samples that will maximize sensitivity. Obviously using a PCR detection methodology on samples that only rarely host the targeted analyte during for example the course of a disease, will most probably cause a down estimate of positivity even if the MDL of the specific assay is very low. Furthermore, when the experimental plan relies on a type of sample that contains very small amounts of the analyte, it should also be expected that reproducibility of PCR results will be poor because of the decreased likelihood that all aliquots of the sample will contain the amplification target [4]. Specifically for pathogens one is also obliged to consider the genetic variations of the DNA region that is used for their detection. This issue can be a source of inconsistency depending on how conserved this region is at the species and strain level, or perhaps even among isolates collected from different geographic areas. If these variations refer to the number of copies of the targeted region they will probably affect the methods MDL. If they refer to the nucleotide sequence of the amplification target, they can affect sensitivity especially if they are located at the annealing sites of the primers [6].

However, what is a very common source of speculation when designing an approach to assess method sensitivity is defining "true positives". This requires the use of what we refer to as the gold standard methodology. For this purpose, microbial isolation in selective media is usually suggested as the safest choice, at least with regard to detection of pathogens. However comparative evaluation of clinical samples by PCR and cultivation often produces discordant results. These are usually associated with the different level of MDL of the two methods, and the fact that the outcome of culture is determined by the viability

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of the pathogen, which does not directly affect PCR results. Admittedly defining true positives based on culture results can be very significant especially if the issue at hand is clinical evaluation, i.e. will my molecular method be able to detect at least all the samples that would be characterized by cultivation as positive? In every other case it would be perhaps more appropriate to use a combination of criteria to define the population of true positives. Obviously one of these criteria could be the result of cultivation but in order to be consistent with the technical specifications of PCR it would be advisable to consider the use of one or more DNA detection methodologies. One rather appealing approach for the assessment of sensitivity of the method under study would be to define true positives based on the results recorded on the same samples using an accredited PCR or real time PCR assay performed preferably in a laboratory that applies them with respect to ISO17025 standards. In short, as opposed to what is in several cases indicated by journal review boards, the selection of the gold standard methodology should be done with consideration to the objective of each experimental design, which means that cultivation should not be considered the only method of reference.

Negative Controls

Use of properly designed negative controls. Though the confirmation of the specificity of the amplification product and the detection of false positive reactions generated by the carry-over effect (passage of amplicons from previous reactions) can be performed rather easily, the same cannot be stated with connection to false negative results. It is exactly because of this why the latter category of false reactions is more likely to compromise the outcome of an investigation that relies on PCR. Notably, the potential impact of undetected false negative reactions to the results that will be recorded and effectively the reliability of the study's final conclusion, is reversibly analogous to the prevalence of the targeted diagnostic indicator.

Detection of false negative reactions relies in most cases on the use of confirmed positive control samples. Ideally the assessment is implemented by the application of a PCR assay targeting an in-house gene to all the samples under study, in order to detect the presence of PCR inhibitors. However this approach does not allow an accurate assessment of each of the samples tested for false negative result generated by fragmentation of the target DNA, poor performance of the thermocycler, or perhaps pipetting errors. The impact of such arbitrary events can be assessed with the use of internal amplification controls (IAC).

The IAC is a DNA molecule that if present in the PCR reaction mixture together with the target region, will produce an amplification band that can be easily distinguished from the one that indicates a positive test result. PCR assays that contain an IAC produce one more band when the result is positive and only a single band when it is negative. In the event that no amplification products are detected, the user has to consider that this may be the result of a false negative reaction. Effectively the incorporation of IACs in PCR increases substantially the reliability of the assessment, which is of obvious significance with connection to research and even more so, diagnostic applications [7].

The IAC can be a host gene or an artificial DNA molecule. In the latter case it can be constructed so it is amplified by the same pair of primers used for the detection of the diagnostic target sequence, or by a second set of primers added in the reaction mixture in the form of a multiplex PCR assay. This is usually the applicable practice when the IAC is a host gene. Admittedly the reaction has to be tuned very carefully, which is not a simple task especially for samples carrying a very small number of target copies. In this case the IAC will present a more effective substrate for amplification, which may generate imbalanced PCR results.

Conclusion

The know-how and the effort that has to be invested for the reliable application of published PCR assays to epidemiology and diagnostic studies, are substantial. Considering the progress recorded this far with connection to the development of PCR-based detection methodologies and the way these are being applied in practice, it becomes rather evident that the need at the moment is to establish strict standardised guidelines. This task however will require a substantial period of time, broad interdisciplinary collaboration and what is very significant, allocation of considerable resources.

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