

Inhibition of Amplification in DNA Amplification Assays For *Chlamydia trachomatis*

National Chlamydia Laboratory Committee
National Infertility Prevention Project

Although increased sensitivity of amplification tests over that for culture for chlamydia has been achieved in recent years, there remains a problem of ill-defined inhibitors to amplification, which are present in some clinical specimens. These inhibitors were first noted when culture positive specimens were PCR negative ¹. Many of the repeat PCR tests after storage of the processed specimens were positive. Most investigators now believe that inhibitors to amplification exist in a small percentage of both urine and cervical specimens²⁻⁴. These inhibitors are thought to be either *labile*, if negative tests later repeat as positive from the same specimen, or *stabile*, if dilution or other treatment, such as DNA purification, is needed to remove the inhibitors. Two studies have reported low levels of inhibitors for the PCR (Roche Amplicor, Branchburg, NJ) assay ranging from 1.8% (women) to 2.6% (men and women) for urine specimens ^{2,3}. These percentages of inhibitors have been directly determined by use of the Roche internal control in the co-amplification assay for chlamydia and gonorrhea. One study reported much higher inhibition (38/200, 19%) for cervical specimens by Roche PCR ⁴.

Various techniques have been investigated to aid in removal or reduction of inhibitors. A 1:10 dilution of the processed specimen (in the Roche transporter) has been used by some investigators. One study found that this dilution reduced inhibition of PCR (Roche) for cervical specimens from 19% to 9.3%, whereas heat treatment (95⁰ C for 10 min.) reduced the inhibition to 9.3% ⁴. A combination of heat treatment and 10-fold dilution of the processed specimen reduced inhibition of PCR from 19% to 4.1% in that study ⁴. Freezing thawing decreased the inhibition to 15.7% and pretreatment at 4⁰C yielded inhibition in 21.2% of specimens ⁴.

Higher pH (≥ 7.5) of the cervical mucosa was partially correlated with decreased inhibition, while presence of blood did not significantly affect inhibition⁴. Delayed processing of more than 72 hours resulted in inhibition of 15.5% compared to inhibition of 27.6% for specimens processed the day of collection or the day after⁴. Using a 1:10 dilution of 2-SP chlamydia transport medium as the swab transporter in that study or centrifugation of the 2SP specimen both resulted in 5.8% of the specimens having inhibition⁴.

In one study, investigators found fewer false negatives, which may be indicative of inhibitors, in swabs, which were transported “dry” to the laboratory and then placed into the Amplicor transport medium⁵. Additionally, it has been demonstrated that adequacy of the cervical specimen with regard to the presence of columnar epithelial cells in the specimen, drastically affects the performance of PCR^{5,6}. The addition of BLOTTO (bovine lacto transfer technique or 10% skim milk) has been reported to attenuate inhibition for research PCR procedures⁷.

The degree to which inhibitors influence the determination of prevalence needs to be further studied. Roche Molecular Systems has addressed this problem by incorporating an internal DNA control amplification and detection assay into their new combination PCR assay for *C. trachomatis* and *N. gonorrhoeae*. This methodology will improve the reliability of diagnostic amplification assays, since inhibited specimens can be diluted, have DNA extracted, or be heated and repeated. The use of the internal control will give a greater degree of confidence in the validity of a negative amplification result. Any amplification test in which inhibitors are problematic should have an internal control.

Inhibition was reported in one recent study comparing Roche Cobas Amplicor and LCR in 2% of male urine specimens and in 20% of female cervical swab specimens using Cobas⁸. Dilution eliminated the inhibition and retesting revealed that all of those cervical swab and male urine specimens to be negative. Although the direct frequency of LCR inhibition could not be determined, 5 true positive male urines were inhibited as they retested positive after either freezing and thawing or dilution for an inhibition rate of 2.5% (5/204)⁸.

In a comparison of PCR, LCR and transcription mediated amplification (TMA) (388 specimens) by Mahoney et al, the prevalence of inhibitors in urine resulting in complete inhibition of amplification was 4.9% for PCR, 2.6% for LCR, and 7.5% for TMA⁹. The majority of inhibition was eliminated by storage overnight at 4C or -70C and diluting 1:10 (84% for PCR, 100% for LCR, and 92% for TMA). Only 2 urine specimens required phenol-chloroform extraction to eliminate inhibition. Urine variables that were independently associated with inhibition of amplification were beta-HCG and crystals for PCR, nitrates for LCR, and hemoglobin, nitrates, and crystals for TMA⁹.

Another way in which investigators have looked for inhibition in specimens is to “spike” the LCR or PCR negative specimen with very small numbers of chlamydia and then retest the specimen. The premise is that a specimen inhibitor would inhibit the repeat test from being positive, even though it contained chlamydia. In a comparison study between PCR and LCR, two PCR positive specimens, who were LCR negative, were investigated for inhibitors by a “spiking experiment, with one being found to contain inhibitors¹⁰. In a study comparing urine LCR to cervical culture in women attending PAP smear clinics, none of 55 urines that were LCR negative were inhibited when spiked with 5 inclusion-forming units of *C. trachomatis*¹¹.

There has been recent interest in the possibility greater levels of inhibition to LCR in the urine from pregnant women, although a direct measure of inhibition was not performed as can be done in the PCR test¹². The urine specimens in this study were shipped at ambient temperatures and this may have compromised the results, others have not found increased inhibition in specimens from pregnant women^{13,14}. Andrews et al. found sensitivity for urine LCR of 82.8% relative to a positive cervical culture or a confirmed positive LCR of the cervix for pregnant women¹⁴. Their sensitivity for LCR on cervical specimens was 96.6%¹⁴. In a study by Gaydos et al. comparing LCR on urine specimens to culture of cervical specimens did not find inhibition to be any more of a problem in pregnant women than in non-pregnant women¹¹. That study reported that 3/65 (4.6%) urine specimens from pregnant women contained inhibitors

by the Roche internal control assay, while none of 55 LCR negative frozen processed urines, which were spiked with chlamydia, were inhibitory. Additionally, only 4 urine specimens were LCR negative and cervical culture positive in that study and none were from pregnant women. Sensitivity and specificity of LCR on urine specimens in that study were 88.6% and 99.7%, respectively. In an ongoing study it has been reported that of 93 pregnant women from a sample of 1333 women, there were no discordant results between cervical culture and urine LCR, which could have been indicative of inhibition (personal communication Agnes Clark, Univ. Washington). Careful removal of all urine from the pellet after centrifugation for LCR testing has been recommended by the manufacturer to aid in reducing inhibition (personal communication, Patricia Plier, Abbott Laboratories). Zurrow et al reported that 50ul of urine on the pellet after processing resulted in an average signal reduction of 34% when tested by LCR, confirming the importance of removing residual urine from pelleted urine specimens ¹⁵. In a further experiment, 179 urine specimens were spiked with a low level of extracted plasmid DNA and 12 (6.7%) were found in which the LCR signal was suppressed by at least 30% compared to plasmid DNA in buffer alone with no urine, indicating the upper limit of inhibitory specimens in that population to be 6.7% ¹⁵.

Reproducibility problems were reported in one PCR study, in which samples were retested by Roche PCR and it was found that six samples initially positive by PCR were negative upon repeat testing and 3 initially negative by PCR were positive when retested ¹⁶. This study was commented on by a Roche scientist, who concluded that particular study was compromised by an unusually high frequency of inhibition in specimens and possibly by specimens that contained low levels of chlamydia organisms ¹⁷. Future use of the internal control in Roche assays will alleviate these problems in the near future. Although the internal control assay is available outside the United States, it is not commercially available in the U.S. ¹⁷. Other manufacturers of amplification tests should consider incorporation of an internal control or other indicators of inhibition in their assays if inhibition has been shown to be a problem. For PCR specimens suspected to contain inhibitors the recommendation to eliminate inhibition is to repeat the DNA amplification assay and/or perform the assay by using a 1:10 dilution followed

by heating of the processed specimen. For testing of urine samples from females by LCR, careful removal of all of the urine from the pellet in the processing step could be recommended as a means to reduce inhibition.

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