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REVIEW

Review of *Chlamydia trachomatis* viability methods: assessing the clinical diagnostic impact of NAAT positive results

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ABSTRACT

Introduction: *Chlamydia trachomatis* (chlamydia) is the most commonly diagnosed bacterial sexually transmitted infection (STI) worldwide. The advancement of molecular techniques has made chlamydia diagnostics infinitely easier. However, molecular techniques lack the information on chlamydia viability. Where in routine diagnostics the detection of chlamydia DNA or RNA might suffice, in other patient scenarios, information on the viability of chlamydia might be essential.

Areas covered: In this review, the authors discuss the specific strengths and limitations of currently available methods to evaluate chlamydia viability: conventional cell culture, messenger RNA (mRNA) detection and viability-PCR (V-PCR).

PubMed and Google Scholar were searched with the following terms: *Chlamydia trachomatis*, Treatment failure, Anal chlamydia, Microbial viability, Culture, Viability-PCR, Messenger RNA, and Molecular diagnostics

Expert commentary: Several techniques are currently available to determine chlamydia viability and thus the clinical relevance of a positive test result in clinical samples. Depending on the underlying research question, all three discussed techniques have their merits when testing for viability. However, mRNA methods show the most promise in determining the presence of a true infection, in case the chlamydia reticulate body can be specifically detected. Further research is needed to understand how to best apply viability testing in current chlamydia diagnostics.

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1. Introduction

Chlamydia trachomatis (chlamydia) is the most common bacterial sexually transmitted infection (STI) globally. A total number of 131 million new cases of chlamydia infections occurred in adults worldwide in 2012 [1]. *Chlamydia trachomatis* strains can be divided into three biovars, based on clinical implications and pathogenesis, and can be further subtyped into serological variants (serovars); the ocular trachoma biovar (serovar A-C), genital biovar (serovar D-K), and the invasive lymphogranuloma venereum (LGV) biovar (serovar L1-L3) [2]. The clinical picture is highly variable, and ranges from virtually no symptoms to symptoms of urethritis, intermenstrual bleeding, and vaginal or urethral discharge [3]. Complications arise mostly in women where chlamydia can result in pelvic inflammatory disease (inflammation of female reproductive organs), which in turn can lead to chronic abdominal pain, ectopic pregnancy and tubal factor infertility [3].

Chlamydia is an obligate intracellular microorganism, and requires its host for essential nutrients. Its life cycle consists of two main stages: a spore-like form which can exist outside host cells and infect new cells, the so-called elementary body (EB), and the replicating, intracellular form known as the reticulate body (RB). The chlamydia life cycle can enter a third stage when exposed to certain stressors, like interferon gamma, penicillin or iron-depletion, where the organism is metabolically active, but does not divide and continues to increase in size. This stage is called an aberrant body (AB) [4].

The diagnosis of chlamydia has changed much in the last decades. Where chlamydia was initially diagnosed using cell-culture, now it is diagnosed via nucleic acid amplification tests (NAATs). Current commercially available NAATs target chlamydia DNA or RNA with high specificity (>99%) and sensitivity (>90%), and are theoretically able to detect a single copy of target sequence [5–8]. Chlamydia testing can be performed using urine, vaginal swabs, cervical swabs, oral swabs and anal swabs. Self-collected samples (i.e. urine, vaginal swabs and anal swabs) have been validated to be equally suitable as compared to specimens collected by a clinician [9–12]. With the advancement of molecular techniques the diagnosis has become easier, but it also exposed the sheer number of people infected; a number which is still increasing to date [1].

The clinical relevance of a chlamydia infection diagnosed with culture is apparent, and the patient will receive immediate treatment. Usually, a chlamydia positive NAAT result will also be interpreted as evidence of a chlamydia infection and thus be

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considered clinically relevant. As a direct consequence the patient will receive treatment. There are, however, patient scenarios where the clinical interpretation of a chlamydia positive NAAT test is unclear. In such cases, it would be beneficial to determine the viability of chlamydia to assess if an actual infection is present. This viability cannot be assessed with the currently used routine NAAT. The viability has been defined as the ability to live, and certain criteria must be met: able to maintain membrane integrity (protection from external environment), active metabolism (self-maintenance/energy uptake and production of proteins) and reproduction (culturability) [13].

In the following paragraphs, three patient scenarios will be described where viability testing could provide new insights regarding the clinical diagnostic impact of chlamydia NAAT positive results.

The first scenario in which the clinical implication of NAAT positive results are unclear is the persistent detection of chlamydia after treatment. Several reports have demonstrated that there is constant or intermittent detection of chlamydia DNA after first-line treatment, even in patient groups with a very low risk of re-infection [14–17]. The clinical relevance of these positive tests is unknown, and it is unclear whether this represents a persistent infection or is due to the detection of remnant DNA originating from dead chlamydia. As a result, a test-of-cure for chlamydia after treatment is currently not recommended [18–20].

The second scenario in which the clinical implication of NAAT positive results are unclear concerns women with a concurrent genital and anal infection. Recent studies have indicated that anal chlamydia infections are much more common in women than was previously thought. Of all women with a genital infection, 33–83% has a concurrent anal infection [21–26], and 71–95% of women with an anal infection has a concurrent genital infection [22,25,27–30]. Notably, the majority of these women did not report anal intercourse or the use of anal toys as a possible source of infection [29]. Possible explanations include underreporting of anal sex, contamination in the laboratory, contamination during self-sampling (e.g. accidentally touching the vagina or perineum before sampling anally), inoculation of the rectum with chlamydia (no infection -yet, but superficial presence of bacteria), and a 'true' infection through auto-inoculation via seepage of infected vaginal secretions [21,25] or via the digestive tract [31,32]. Here, it needs to be determined if the detected bacteria are alive, and if so, whether this represents a true (intracellular) infection or not. These answers are particularly critical as the treatment for a genital infection differs from the treatment of an anal infection in most countries [18–20].

Finally, for the third scenario, it has been demonstrated that chlamydia infections may clear spontaneously, or they may persist for several months or years [33–35]. It is currently unclear exactly what determines if an infection will be resolved, but it is known that infections with a low bacterial load are cleared more easily [36,37]. These studies were performed with NAAT, so it is unknown whether low loads are remains of bacteria already eliminated by the immune system or if they are viable bacteria. Also in this scenario the assessment of the viability of chlamydia could provide crucial answers [38].

The abovementioned cases are encountered in daily practice and illustrate the need for viability assessment. Several methods have been proposed to evaluate the viability of microorganisms based on the above mentioned viability criteria. Here, we review the specific strengths and limitations of currently available methods to evaluate chlamydia viability: conventional cell culture, mRNA detection, and viability-PCR. In all three methods, specimen collection and transport conditions should be optimized to retain chlamydia viability until laboratory assessment.

2. Chlamydia viability assays

2.1. Culture

Bacterial culture is the most conventional method applied in medical microbiology laboratories for the isolation of many pathogens. Furthermore, culture methods are extensively applied to demonstrate the ability (or inability) of a microorganism to multiply in predetermined culture medium and laboratory conditions. Also for viability assessment in chlamydia, culturing is still the gold-standard methodology, as it assesses all necessary criteria (membrane integrity, active metabolism and reproduction).

Chlamydia is an obligatory intracellular bacterium that requires host cells for reproduction, which makes it a challenging organism to cultivate. Chlamydia was initially propagated in embryonated hen eggs and later in a more convenient tissue culture system [39]. Today, most commonly used host cell types for the propagation of chlamydia are the HeLa-229 cells derived from human cervical adenocarcinoma and the McCoy cells derived from mouse fibroblasts [40]. Host cell infection is usually facilitated by centrifugation assisted inoculation or pretreating host cells with diethylaminoethyl-dextran, both altering host cell membranes to enhance EB adhesion and entry [41]. To further increase culture sensitivity, culture medium of inoculated monolayers is supplemented with cyclohexamide. Cyclohexamide inhibits eukaryotic protein synthesis but has no effect on prokaryotes, allowing chlamydia to outcompete host cells for the acquisition of nutrients and preventing host-cell overgrowth [42,43]. Following an incubation period of 48–72 h, the infected cells are usually fixed and stained with fluorescently labeled monoclonal antibodies targeting chlamydia genus specific lipopolysaccharides or biovar-specific targets such as the major outer membrane protein (MOMP) [44]. Chlamydia culture has mainly been correlated to clinical parameters like symptoms, but has also been used as a test-of-cure [17].

2.1.1. Strengths and limitations

The observation of typical intracytoplasmic inclusions together with the specific fluorescently stained chlamydia contributes to the high specificity of culture methods, which approaches 100% specificity (Table 1). Therefore, chlamydia culture followed by immunofluorescence microscopic evaluation is the traditional gold standard method for the diagnosis of chlamydia infection and represents the definitive method for the evaluation of viability [45]. However, traditional culture methods are also known for their relative low sensitivity compared to modern NAATs, which has been reported to be as low as 50% [46,47]. This sensitivity may be further reduced by culture overgrowth by commensal bacteria [47]. Recently, however,

Table 1. Overview of methods for the evaluation of chlamydia viability.

Method	Principle	Advantages	Disadvantages
Culture	Sample inoculation on tissue culture monolayer. Chlamydia viability assessment by membrane integrity, active metabolism and bacterial reproduction.	–Highly specific for viability assessment	–Low sensitivity –High turnaround times –Highly resources demanding –Only performed in specialized laboratories –Viability based on membrane integrity alone –Unknown membrane disintegration time after death –Cannot differentiate between specific life stages (e.g. chlamydial EBs and RBs)
Viability-PCR	Sample pretreatment with membrane impermeable DNA binding dyes followed by quantitative PCR, resulting in the selective quantification of DNA originating from chlamydia with intact membranes.	–Fast and easy to use –Easy to implement in molecular based laboratory testing –High sensitivity and specificity for the detection of target organism without needing additional equipment –Quantification of viable organisms	
Messenger RNA detection	Reverse transcriptase quantitative PCR to detect short-lived mRNA molecules, representing metabolically active microorganisms.	–High sensitivity and specificity for the detection of target organism –Can be implement in molecular based laboratory testing without needing additional equipment –Active metabolism as a proxy for viability –Life stage specific transcripts can be targeted	–Chemically labile RNA molecules require cooled workflow –Genomic DNA contamination can lead to false positive results –Unknown duration of transcript persistence after death –Transcripts persistence after death can vary between gene targets

Shao *et al*. demonstrated an increased culture sensitivity by performing additional *in vitro* passages compared to standard protocols and reported a culture sensitivity of 80% after three blind culture passages [48], but this will lengthen the time to diagnosis even more. Culture methods have mostly long turnaround times of at least 24 h and up to 3 days post infection without additional *in vitro* passages and with its labor intensive and demanding procedure, chlamydia culturing is not available in most routine laboratory settings.

2.2. Detection of messenger-RNA

The introduction of NAAT expanded the toolbox of medical microbiology laboratories to diagnose chlamydia infections with high sensitivity and specificity. However, NAAT cannot differentiate between DNA originating from viable and dead bacteria. In contrast, messenger-RNA (mRNA) molecules are known to be short-lived, with half-lives in the range of minutes, and thus detection of mRNA represents the presence of viable (metabolically active) bacteria [49].

RNA-based approaches have been applied in various research fields, on a wide variety of microorganisms, and by a diversity of laboratory methods, of which reverse transcriptase (RT)-PCR is the most commonly applied method to evaluate gene expression levels [50–52]. The RT-PCR method globally consists of two stages: first the extracted RNA is converted to complementary DNA (cDNA) using reverse transcriptase. Subsequently, the cDNA serves as template for amplification and detection of target genes by PCR or quantitative PCR.

Several approaches have been described which implemented detection of mRNA as a marker for chlamydia viability. Generally, these approaches can be used to address two important knowledge gaps regarding the diagnosis of an ongoing infection: it is unknown (i) whether the detected chlamydia are viable or not and (ii) in which stage of the life cycle (EB, RB or *AB*) the detected chlamydia are.

First, to be able to use mRNA as a marker of viability it is necessary to monitor the decay rates of mRNA in chlamydia. Ferreira *et al*. [53] demonstrated a large variability in the decay rate (expressed as a half-life time [$t^{1/2}$]) of chlamydia mRNA, with a maximum of ~5000 min (majority <65 min), which varied greatly per chlamydia strain. This contrasts with that of other (facultative intracellular) bacteria which have shown a maximum of ~35 min. In spite of the variable decay rate, researchers have implemented mRNA as a viability marker to monitor anti-chlamydia treatment efficiency [54–59]. Studies on chlamydia susceptibility usually applied multiple targets encoding heat-shock protein 60 (HSP60; *gro*EL), unprocessed 16S rRNA (16S precursor rRNA), or genes encoding for one of the outer membrane proteins (omcA, omcB). To our knowledge, there are no studies that have applied chlamydia mRNA as a test of cure in clinical samples.

Regarding the second knowledge gap, mRNA detection has been used to study gene expression during the different life stages of chlamydia [60–62]. During the chlamydia life cycle, three temporal stages of gene expression have become apparent: Early expressed genes (detection at 2 h post infection) encode for proteins involved in EB to RB

differentiation, such as 16S rRNA and *rpo*B (β subunit of RNA polymerase). Typical genes of which expression is initiated at mid-cycle stage (6-12h post infection) encode for proteins involved in growth and replication of RBs, such as *omp*A (encoding for MOMP) and *inc*A (inclusion membrane protein). Late-cycle genes (20 h post infection) typically encode for proteins involved in RB to EB differentiation, such as *hct*A (histone H1 homologue) and *omc*B (outer membrane protein). In ABs, proteins for division and replication are downregulated (such as *omc*A and *omc*B) while stress-proteins (such as HSP60) are upregulated [63].

2.2.1. Strengths and limitations

The main strengths of the use of mRNA for determination of chlamydia viability are the shorter time to results when compared to culture as well as the high specificity and sensitivity of this method. Furthermore, this approach can detect the transcription of life-stage-specific genes, which is not possible with other methods.

However, due to its chemically labile composition, working with mRNA as a template can be challenging. RNA is more susceptible for degradation by heat than DNA and should be kept on ice during the complete workflow. Moreover, RNA can be degraded easily by ribonuclease (RNAse) activity, thus the use of RNAse inhibitors is highly recommended [64]. In addition, caution must be taken when interpreting results, as RNA purification methods do not always completely eliminate genomic DNA which can result in false positive results. Furthermore, there is no consensus on how long mRNA persists after chlamydia death, and how much this differs between different transcripts [53].

2.3. Viability-PCR

NAATs are known to amplify the targeted nucleic acids without discriminating between DNA originating from viable or dead bacteria. However, implementation of membrane-impermeable DNA intercalating dyes (e.g. propidium monoazide; PMA) as a sample preparation step before conducting NAAT has shown to be a promising approach for the selective detection of viable bacteria. In this approach, called viability-PCR (V-PCR), the distinction between viable and dead bacteria is based on membrane integrity. V-PCR consists of incubating the sample with a DNA intercalating dye which can only pass compromised membranes. Upon photoactivation, the DNA intercalating dye irreversibly binds to the exposed DNA, which in turn prevents amplification by PCR [65–67]. Subsequently, the observed difference in bacterial load between the PMA treated and untreated sample correlates with the bacterial viability in the original sample. The amount of dead bacteria is greater with increasing differences in the observed cycle of quantification values (C_q).

Nogva *et al*. utilized ethidium monoazide (EMA) as a sample treatment agent, which upon photoactivation binds covalently to DNA and subsequently prevents amplification of exposed DNA [66,68]. Although this method showed promising results in different bacterial species [65,69–71], EMA treatment can result in loss of more than half of the genomic DNA of viable cells [67]. Therefore, the newly developed viability dye propidium monoazide (PMA) has been introduced as an alternative viability dye [67]. Since, V-PCR has been successfully applied in the detection of viable microorganisms in clinical [72], food [73–75], and environmental samples [76–78].

To this date, only one study by our group implemented the V-PCR method to assess chlamydia viability in clinical samples [79]. V-PCR showed that the majority (64%) of vaginal swabs taken before treatment, contained 10% or more viable chlamydia. However, one–third (34%) of the samples contained <1% viable bacteria [79]. Furthermore, it has been demonstrated that V-PCR results correlate with the results of chlamydia culture [*Manuscript in preparation*]. While these studies have shown that V-PCR is a feasible and valid method to assess chlamydia viability in anal and genital swab samples, validation of this method for other sample types is still needed. This method is currently being applied in a large multicenter follow-up study in the Netherlands (FemCure study) to study chlamydia viability in women after treatment [80].

2.3.1. Strengths and limitations

V-PCR has been successfully implemented as a chlamydia viability assay, it combines the ability to distinguish viable from dead bacteria with the high sensitivity, specificity, and short turnaround time of NAAT. Furthermore, V-PCR is an easy to use method, as only a short sample preparation step is needed in addition to the normal routine workflow.

The main limitation of this approach is that viability is solely based on membrane integrity. It has been demonstrated that bacterial viability can be lost without a direct effect on membrane permeability, resulting in a potential overestimation of viability. Cangelosi *et al*. [81] reviewed how surfactants and oxidative disinfectants directly results in membrane damage, while antibiotics indirectly cause loss of membrane integrity over hours or days. Furthermore, there is a risk of underestimation of viability when the bacterial load in a sample is close to the limit of detection. V-PCR is based on the C_q difference in two samples, where the removal of free DNA by PMA-treatment may increase the resulting C_q value. In samples with a low bacterial load however, this shift in C_q value may exceed the detection limit of the qPCR, resulting in the absence of a signal. Consequently, it is unclear whether the sample contains an undetectable amount of viable bacteria, or no viable bacteria at all.

3. Conclusion

To date, three different methodologies are being used in research settings to assess chlamydia viability: i.e. culture, detection of mRNA and V-PCR. None of these methods are used in practice yet as a test-of-cure or to assess viability for patient management.

Chlamydia culture represents a highly specific method for chlamydia viability, and is still the gold standard. It assesses all viability-criteria simultaneously: membrane integrity, active metabolism and reproduction. An additional advantage of culture techniques is the possibility of downstream analysis on isolated clinical strains such as antimicrobial resistance monitoring and genotyping. Therefore, maintaining laboratory expertise in chlamydia culture is still needed.

The detection of mRNA shows promising results as a potential marker for chlamydia viability, as it can be used to assess active metabolism. Its half-life is relatively short (minutes-hours) and as such is regarded as a marker of viability and it can be used to establish the presence of certain stages in the chlamydia life cycle. However, the sample handling can be challenging. Future studies should investigate the mRNA degradation time, to establish when mRNA can reliably be used to test viability, and which targets are most appropriate.

In recent years, V-PCR has been implemented for the viability assessment in a wide variety of organisms including chlamydia. V-PCR demonstrates an intact membrane as a proxy of bacterial viability, but here too it needs to be established at what time chlamydia membranes become permeable after loss of viability, and if this differs between antibiotics.

In short, mRNA and V-PCR are promising alternatives for traditional cell culture when assessing chlamydia viability.

4. Expert commentary

The advancement of molecular techniques has made the diagnosis of chlamydia infections infinitely easier when compared to the time when chlamydia diagnoses were based on tissue culture. However, the drawback of this development is the lack of information on chlamydia viability. Whereas in routine diagnostics, the detection of chlamydia DNA or RNA might suffice, in other patient scenarios, information on the viability of chlamydia might be essential. In fact, there are overall two knowledge gaps that can be identified regarding chlamydia viability: it is unknown (i) whether the detected chlamydia are viable or dead and (ii) if the detection of a chlamydia genome represents a true infection or not. When addressing issues such as the persistence of chlamydia after treatment or in the case of a test-of-cure, the answer to the question whether the bacteria are alive, will by itself provide the necessary information. For example, when replicating bacteria are detected after treatment this is strongly indicative of treatment failure. All three currently used techniques to assess chlamydia viability (i.e. cell culture, mRNA, and V-PCR) can effectively address this issue, provided sampling and transport conditions has not diminished potential chlamydia viability. The choice of technique ultimately depends on the availability of culture facilities and laboratory expertise.

There are however, other clinical questions that need additional information in order to be answered. For example, when considering the clinical relevance of anal chlamydia detection in women, the answer lies in establishing whether women are indeed infected with chlamydia or whether NAAT positivity represents the superficial presence of chlamydia. Establishing that chlamydia is alive, will only provide part of the answer and proof or indication of the presence of infection is needed, rather than the presence of living bacteria. There are several approaches one could envision to indicate the presence of infection. Using immunofluorescent staining to detect intracellular chlamydia (i.e. detection of RBs) in tissue biopsies would provide the ultimate proof that a chlamydia infection is present. However, due to its invasiveness and low sensitivity this method is rarely used. The specific detection of RBs using other methods, could be equally valuable. The most promising method to date is the detection of life-stage specific mRNA. Different studies have already shown differential expression of certain mRNAs *in vitro* [60–62]. However, to our knowledge, this method has not yet been able to make the distinction between RB and EB, or specifically detect RBs, in clinical samples.

A completely different approach could be to detect human markers of infection. However, there are to date no specific biomarkers which can be used to prove the presence of a chlamydia infection, but mucosal antibodies have been investigated in this light [82].

In short, several techniques exist to determine viability and thus the clinical relevance of a positive NAAT result in clinical samples. When the question of viability arises it is important to distinguish two aspects: the chlamydia viability and the presence or absence of a chlamydia infection. Each aspect can be investigated by a distinct technique. All three techniques have their merits when testing for viability, but mRNA shows the most promise in determining the presence of a true infection, in case RB can be specifically detected.

5. Five-year view

The field of chlamydia viability testing has only recently gained more attention, when clinically relevant questions beyond the scope of routine diagnostics, needed to be answered. When foreseeing the future in this growing area, there are two important issues to consider. First of all, which technologies can be further developed or might enter the field, and second of all, how can current or future technologies for chlamydia viability testing impact diagnosis and treatment policies.

Looking at which technologies might impact the field in the future, it is clear that the developments regarding V-PCR and mRNA detection are highly promising. Both methods have the potential to be automated, which is a valuable aspect for the implementation in a high throughput workflow or a point-of-care setting. However, several questions still need to be answered: for V-PCR more information is needed about when the chlamydia membrane becomes permeable. While in the area of mRNA detection results on how long mRNA persists in the human body need to be corroborated, and further research is needed to show which gene(s) would be most suitable as a marker of chlamydia viability as well as for differentiation or detection of the specific chlamydia life-stages in clinical samples. While these technologies have already shown much promise in the field of chlamydia viability, other techniques could also be employed in the future. Several techniques have been successfully applied to other microorganisms. Such techniques include, the use of propidium iodide in combination with flow cytometry, and bioluminescence assays to determine ATPase activity, or the use of human local tissue markers such as antibodies [82] or excreted vesicles [83,84].

Regarding the future of viability testing in chlamydia, it is also important to consider how viability testing might impact the field of chlamydia diagnosis and treatment. In this review we have addressed three patient scenarios. Looking at the clinical relevance of anal chlamydia detection in women, it is clear that studies assessing whether this detection indicates true infections can directly impact patient care. According to current guidelines, women are only tested for anal chlamydia when showing symptoms or when indicating having anal sex

[18–20]. However, studies have shown that anal chlamydia detection is as common in women without these indications as it is in women with such testing indications [21–26,28]. When anal chlamydia detection is shown to be associated with true infections, anal NAAT testing would likely become part of the recommended testing routine.

It is known that chlamydia infections can be subject to natural clearance. A recent study using V-PCR suggested that a significant proportion of samples from chlamydia positive women attending an STI clinic showed less than 1% viable chlamydia [79]. Currently, all NAAT-positive patients are receiving antibiotic treatment regardless of the chlamydia viability status. Perhaps, in the future when rapid viability testing methods such as mRNA detection or V-PCR are more widely implemented, patients with no evidence of viable chlamydia will no longer require antibiotic therapy.

Finally, viability testing could have an even stronger impact in the follow-up of persistent chlamydia detection after treatment. Currently, performing a test-of-cure is not recommended due to the prolonged detection of chlamydia DNA or RNA after treatment. If, research would show that viable chlamydia can be detected in such cases, treatment regimens could be directly impacted as this is a strong indication of treatment failure. Furthermore, performing a test-of-cure could be introduced when chlamydia detection could be combined with viability testing. With the introduction of mRNA detection or V-PCR, sensitive methods have become available that can be implemented in high-throughput routine diagnostics in the near future.

Key issues

- Bacterial viability is based on three criteria: membrane integrity, active metabolism, and reproduction, which can be assessed through culture.
- *Chlamydia trachomatis* cell culture has a notoriously low sensitivity, which is why current diagnostics is based on nucleic acid amplification tests (NAAT). Because NAAT cannot make the distinction between viable and non-viable bacteria, alternative methods are being investigated, such as messenger RNA (mRNA) or viability-PCR (V-PCR).
- mRNA is produced by metabolically active bacteria, and has been used to differentiate different life stages of the chlamydia life cycle. However, it is technically demanding, and it is unknown how long mRNA can persist in the human body after chlamydia death.
- V-PCR prevents the amplification of bacteria with compromised membranes, thus providing a measure of the viable bacteria in a sample. It has been shown to correlate to chlamydia viability *in vitro*, but needs to be validated in larger clinical studies. Here too, it is unknown how long membranes remain intact in the human body after chlamydia death.
- Chlamydia viability assays may in the future be applied as a molecular test-of-cure after treatment, or to assess the viability in diagnostic samples. Differentiating elementary bodies from reticulate bodies could provide valuable insights into the relevance of positive anal chlamydia NAATs.
- With the introduction of mRNA detection or V-PCR, sensitive methods have become available that can be implemented in high-throughput routine diagnostics in the near future.

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References

Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

1. Newman L, Rowley J, Vander Hoorn S, et al. Global estimates of the prevalence and incidence of four curable sexually transmitted infections in 2012 based on systematic review and global reporting. PloS One. 2015;10(12):e0143304. PubMed PMID: 26646541; PubMed Central PMCID: PMC4672879.
2. Elwell C, Mirrashidi K, Engel J. Chlamydia cell biology and pathogenesis. Nat Rev Microbiol. 2016 Jun;14(6):385–400. PubMed PMID: 27108705; PubMed Central PMCID: PMC4886739.
3. Bennett J, Dolin R, Blaser M. Mandell, Douglas, and Bennett's principles and practice of infectious diseases. 8th ed. Philadelphia (PA): Elsevier Saunders; 2015.
4. Wyrick PB. Chlamydia trachomatis persistence in vitro: an overview. J Infect Dis. 2010 Jun 15;201 Suppl 2:S88–S95. PubMed PMID: 20470046; PubMed Central PMCID: PMC2878585.
5. Gaydos CA, Quinn TC, Willis D, et al. Performance of the APTIMA combo 2 assay for detection of Chlamydia trachomatis and Neisseria gonorrhoeae in female urine and endocervical swab specimens. J Clin Microbiol. 2003 Jan;41(1):304–309. PubMed PMID: 12517865; PubMed Central PMCID: PMC149571.
6. Gaydos CA, Theodore M, Dalesio N, et al. Comparison of three nucleic acid amplification tests for detection of Chlamydia trachomatis in urine specimens. J Clin Microbiol. 2004 Jul;42(7):3041–3045. PubMed PMID: 15243057; PubMed Central PMCID: PMC446239.
7. Van Der Pol B, Ferrero DV, Buck-Barrington L, et al. Multicenter evaluation of the BDProbeTec ET System for detection of Chlamydia trachomatis and Neisseria gonorrhoeae in urine specimens, female endocervical swabs, and male urethral swabs. J Clin Microbiol. 2001 Mar;39(3):1008–1016. PubMed PMID: 11230419; PubMed Central PMCID: PMC87865.
8. Van Der Pol B, Quinn TC, Gaydos CA, et al. Multicenter evaluation of the AMPLICOR and automated COBAS AMPLICOR CT/NG tests for detection of *Chlamydia trachomatis*. J Clin Microbiol. 2000 Mar;38 (3):1105–1112. PubMed PMID: 10699004; PubMed Central PMCID: PMC86350.
9. Hoebe CJ, Rademaker CW, Brouwers EE, et al. Acceptability of self-taken vaginal swabs and first-catch urine samples for the diagnosis of urogenital *Chlamydia trachomatis* and *Neisseria gonorrhoeae* with an amplified DNA assay in young women attending a public health sexually transmitted disease clinic. Sex Transm Dis. 2006 Aug;33(8):491–495. PubMed PMID: 16547452.
10. Moncada J, Schachter J, Liska S. Evaluation of self-collected glans and rectal swabs from men who have sex with men for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* by use of nucleic acid amplification tests. J Clin Microbiol. 2009 Jun;47 (6):1657–1662. PubMed PMID: 19369445; PubMed Central PMCID: PMC2691064.
11. van der Helm JJ, Hoebe CJ, van Rooijen MS, et al. High performance and acceptability of self-collected rectal swabs for diagnosis of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in men who have sex with men and women. Sex Transm Dis. 2009 Aug;36 (8):493–497. PubMed PMID: 19617869.

12. van Dommelen L, Dukers-Muijrers N, van Tiel FH, et al. Evaluation of one-sample testing of self-obtained vaginal swabs and first catch urine samples separately and in combination for the detection of *Chlamydia trachomatis* by two amplified DNA assays in women visiting a sexually transmitted disease clinic. Sex Transm Dis. 2011 Jun;38(6):533–535. PubMed PMID: 21217415.
13. Bogosian G, Bourneuf EV. A matter of bacterial life and death. EMBO Rep. 2001 Sep;2(9):770–774. PubMed PMID: 11559589; PubMed Central PMCID: PMC1084037.
14. Dukers-Muijrers NH, Morré SA, Speksnijder A, et al. Chlamydia trachomatis test-of-cure cannot be based on a single highly sensitive laboratory test taken at least 3 weeks after treatment. PloS One. 2012;7(3):e34108. PubMed PMID: 22470526; PubMed Central PMCID: PMC3314698.
15. Dukers-Muijrers NH, Speksnijder AG, Morré SA, et al. Detection of anorectal and cervicovaginal Chlamydia trachomatis infections following azithromycin treatment: prospective cohort study with multiple time-sequential measures of rRNA, DNA, quantitative load and symptoms. PloS One. 2013;8(11):e81236. PubMed PMID: 24278400; PubMed Central PMCID: PMC3835673.
16. Renault CA, Israelski DM, Levy V, et al. Time to clearance of *Chlamydia trachomatis* ribosomal RNA in women treated for chlamydial infection. Sex Health. 2011 Mar;8(1):69–73. PubMed PMID: 21371385.
17. Versteeg B, Bruisten SM, Heijman T, et al. Monitoring therapy success of urogenital *Chlamydia trachomatis* infections in women: a prospective observational cohort study. PloS One. 2017;12(9):e0185295. PubMed PMID: 28934342; PubMed Central PMCID: PMC5608402.
• **Used cell culture to assess if a positive RNA- or DNA-based NAAT after treatment indicates the presence of viable C. trachomatis.**
18. Lanjouw E, Ouburg S, de Vries HJ, et al. 2015 European guideline on the management of *Chlamydia trachomatis* infections. Int J STD AIDS. 2016 Apr;27(5):333–348. PubMed PMID: 26608577.
19. Nwokolo NC, Dragovic B, Patel S, et al. 2015 UK national guideline for the management of infection with *Chlamydia trachomatis*. Int J STD AIDS. 2016 Mar;27(4):251–267. PubMed PMID: 26538553.
20. Workowski KA, Bolan GA, Centers for Disease C, et al. Sexually transmitted diseases treatment guidelines, 2015. MMWR Recomm Rep. 2015 Jun 5;64(RR–03):1–137. PubMed PMID: 26042815.
21. Ding A, Challenor R. Rectal Chlamydia in heterosexual women: more questions than answers. Int J STD AIDS. 2014 Jul;25(8):587–592. PubMed PMID: 24352134.
22. Dukers-Muijrers NH, Schachter J, van Liere GA, et al. What is needed to guide testing for anorectal and pharyngeal *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in women and men? Evidence and opinion. BMC Infect Dis. 2015 Nov 17;15:533. PubMed PMID: 26576538; PubMed Central PMCID: PMC4650297.
23. Peters RP, Dubbink JH, van der Eem L, et al. Cross-sectional study of genital, rectal, and pharyngeal Chlamydia and gonorrhea in women in rural South Africa. Sex Transm Dis. 2014 Sep;41(9):564–569. PubMed PMID: 25118973.
24. van Liere GA, Hoebe CJ, Niekamp AM, et al. Standard symptom- and sexual history-based testing misses anorectal *Chlamydia trachomatis* and *Neisseria gonorrhoeae* infections in swingers and men who have sex with men. Sex Transm Dis. 2013 Apr;40(4):285–289. PubMed PMID: 23486492.
25. van Liere GA, Hoebe CJ, Wolffs PF, et al. High co-occurrence of anorectal chlamydia with urogenital chlamydia in women visiting an STI clinic revealed by routine universal testing in an observational study; a recommendation towards a better anorectal chlamydia control in women. BMC Infect Dis. 2014 May 19; 14:274. PubMed PMID: 24885306; PubMed Central PMCID: PMC4032161.
• **Study that assessed the high co-occurrence of genital and anorectal chlamydia infections in women. It shows that the majority of anorectal chlamydia infections are diagnosed in women who reported no anal sex or symptoms.**
26. Dubbink JH, de Waaij DJ, Bos M, et al. Microbiological characteristics of *Chlamydia Trachomatis* and *Neisseria Gonorrhoeae* infections in South African women. J Clin Microbiol. 2016 Jan;54(1):200–203. PubMed PMID: 26511740; PubMed Central PMCID: PMC4702750.
27. Dewart CM, Bernstein KT, DeGroote NP, et al. Prevalence of rectal chlamydial and gonococcal infections: a systematic review. Sex Transm Dis. 2017 Nov 1. DOI:10.1097/OLQ.0000000000000754. PubMed PMID: 29465688.
28. Musil K, Currie M, Sherley M, et al. Rectal chlamydia infection in women at high risk of chlamydia attending Canberra Sexual Health Centre. Int J STD AIDS. 2016 Jun;27(7):526–530. PubMed PMID: 25957326.
29. Chandra NL, Broad C, Folkard K, et al. Detection of Chlamydia trachomatis in rectal specimens in women and its association with anal intercourse: a systematic review and meta-analysis. Sex Transm Infect. 2018 Feb 3. DOI:10.1136/sextrans-2017-053161. PubMed PMID: 29431148.
30. van Liere G, Dukers-Muijrers N, Levels L, et al. High proportion of anorectal *Chlamydia trachomatis* and *Neisseria gonorrhoeae* after routine universal urogenital and anorectal screening in women visiting the sexually transmitted infection clinic. Clin Infect Dis. 2017 Jun 15;64(12):1705–1710. PubMed PMID: 28369227.
31. Rank RG, Yeruva L. Hidden in plain sight: chlamydial gastrointestinal infection and its relevance to persistence in human genital infection. Infect Immun. 2014 Apr;82(4):1362–1371. PubMed PMID: 24421044; PubMed Central PMCID: PMC3993372.
32. Yeruva L, Spencer N, Bowlin AK, et al. Chlamydial infection of the gastrointestinal tract: a reservoir for persistent infection. Pathog Dis. 2013 Aug;68(3):88–95. PubMed PMID: 23843274; PubMed Central PMCID: PMC3751173.
33. Geisler WM. Duration of untreated, uncomplicated *Chlamydia trachomatis* genital infection and factors associated with chlamydia resolution: a review of human studies. J Infect Dis. 2010 Jun 15;201 Suppl 2:S104–13. PubMed PMID: 20470048.
34. Morre SA, van den Brule AJ, Rozendaal L, et al. The natural course of asymptomatic *Chlamydia trachomatis* infections: 45% clearance and no development of clinical PID after one-year follow-up. Int J STD AIDS. 2002 Dec;13 Suppl 2:12–18. PubMed PMID: 12537719.
35. Molano M, Meijer CJ, Weiderpass E, et al. The natural course of *Chlamydia trachomatis* infection in asymptomatic Colombian women: a 5-year follow-up study. J Infect Dis. 2005 Mar 15;191 (6):907–916. PubMed PMID: 15717266; eng.
36. Dirks JA, Van Liere GA, Bogers S, et al. Natural course of *Chlamydia trachomatis* bacterial load in the time interval between screening and treatment in anogenital samples. PloS One. 2015;10(12): e0145693. PubMed PMID: 26713628; PubMed Central PMCID: PMC4701017.
37. van Rooijen MS, Schim van der Loeff MF, Morré SA, et al. Spontaneous pharyngeal *Chlamydia trachomatis* RNA clearance. A cross-sectional study followed by a cohort study of untreated STI clinic patients in Amsterdam, The Netherlands. Sex Transm Infect. 2015 May;91(3):157–164. PubMed PMID: 25237127.
38. Vodstrcil LA, McIver R, Huston WM. The epidemiology of *Chlamydia trachomatis* organism load during genital infection: a systematic review. J Infect Dis. 2015 May 15;211(10):1628–1645. PubMed PMID: 25492913.
•• **Systematic review that investigated the correlation between chlamydia load and clinical presentation, indicating that the assessment of viable organism load may be more relevant than total load.**
39. Gordon FB, Harper IA, Quan AL, et al. Detection of Chlamydia (Bedsonia) in certain infections of man. I. Laboratory procedures: comparison of yolk sac and cell culture for detection and isolation. J Infect Dis. 1969 Oct;120(4):451–462. PubMed PMID: 4186430.
40. Evans RT, Woodland RM. Detection of chlamydiae by isolation and direct examination. Br Med Bull. 1983 Apr;39(2):181–186. PubMed PMID: 6191821.
41. Rota TR, Nichols RL. *Chlamydin trachomatis* in cell culture. I. Comparison of efficiencies of infection in several chemically defined media, at various pH and temperature values, and after exposure to diethylaminoethyl-dextran. Appl Microbiol. 1973 Oct;26(4):560–565. PubMed PMID: 4751801; PubMed Central PMCID:PMC379848.
42. Benes S, McCormack WM. Comparison of methods for cultivation and isolation of *Chlamydia trachomatis*. J Clin Microbiol. 1982

Nov;16(5):847–850. PubMed PMID: 6185530; PubMed Central PMCID: PMC272489.
43. Ripa KT, Mardh PA. Cultivation of *Chlamydia trachomatis* in cycloheximide-treated mccoy cells. J Clin Microbiol. 1977 Oct;6(4):328–331. PubMed PMID: 562356; PubMed Central PMCID: PMC274768.
44. Scidmore MA. Cultivation and laboratory maintenance of *Chlamydia trachomatis*. Curr Protoc Microbiol. 2005 Jul:Chapter 11:Unit 11A 1. DOI:10.1002/9780471729259.mc11a01s00. PubMed PMID: 18770550.
45. Barnes RC. Laboratory diagnosis of human chlamydial infections. Clin Microbiol Rev. 1989 Apr;2(2):119–136. PubMed PMID: 2650858; PubMed Central PMCID: PMC358106.
46. Thejls H, Gnarpe J, Gnarpe H, et al. Expanded gold standard in the diagnosis of *Chlamydia trachomatis* in a low prevalence population: diagnostic efficacy of tissue culture, direct immunofluorescence, enzyme immunoassay, PCR and serology. Genitourin Med. 1994 Oct;70(5):300–303. PubMed PMID: 8001937; PubMed Central PMCID: PMC1195269.
47. Meyer T. Diagnostic procedures to detect *Chlamydia trachomatis* infections. Microorganisms. 2016 Aug 5;4(3). PubMed PMID: 27681919; PubMed Central PMCID: PMC5039585. DOI:10.3390/microorganisms4030025
48. Shao L, Guo Y, Jiang Y, et al. Sensitivity of the standard *Chlamydia trachomatis* culture method is improved after one additional in vitro passage. J Clin Lab Anal. 2016 Sep;30(5):697–701. PubMed PMID: 26987564.
49. Laalami S, Zig L, Putzer H. Initiation of mRNA decay in bacteria. Cell Mol Life Sci. 2014 May;71(10):1799–1828. PubMed PMID: 24064983; PubMed Central PMCID: PMC3997798.
50. Cenciarini-Borde C, Courtois S, La Scola B. Nucleic acids as viability markers for bacteria detection using molecular tools. Future Microbiol. 2009 Feb;4(1):45–64. 10.2217/17460913.4.1.45. PubMed PMID: 19207099.
• **Review providing an overview of DNA and RNA detection methods and targets for viability assessment.**
51. Keer JT, Birch L. Molecular methods for the assessment of bacterial viability. J Microbiol Methods. 2003 May;53(2):175–183. PubMed PMID: 12654489.
52. VanGuilder HD, Vrana KE, Freeman WM. Twenty-five years of quantitative PCR for gene expression analysis. Biotechniques. 2008 Apr;44(5):619–626. PubMed PMID: 18474036.
53. Ferreira R, Borges V, Borrego MJ, et al. Global survey of mRNA levels and decay rates of *Chlamydia trachomatis* trachoma and lymphogranuloma venereum biovars. Heliyon. 2017 Jul;3(7):e00364. PubMed PMID: 28795162; PubMed Central PMCID: PMC5541142.
• **Key study investigating the global decay rate of mRNA levels in chlamydia.**
54. Peuchant O, Duvert JP, Clerc M, et al. Effects of antibiotics on *Chlamydia trachomatis* viability as determined by real-time quantitative PCR. J Med Microbiol. 2011 Apr;60(Pt 4):508–514. PubMed PMID: 21183597.
55. Mpiga P, Ravaoarinoro M. Effects of sustained antibiotic bactericidal treatment on *Chlamydia trachomatis*-infected epithelial-like cells (HeLa) and monocyte-like cells (THP-1 and U-937). Int J Antimicrob Agents. 2006 Apr;27(4):316–324. PubMed PMID: 16527461.
56. Storm M, Gustafsson I, Herrmann B, et al. Real-time PCR for pharmacodynamic studies of *Chlamydia trachomatis*. J Microbiol Methods. 2005 Jun;61(3):361–367. PubMed PMID: 15767012.
57. Dreses-Werringloer U, Padubrin I, Jurgens-Saathoff B, et al. Persistence of *Chlamydia trachomatis* is induced by ciprofloxacin and ofloxacin in vitro. Antimicrob Agents Chemother. 2000 Dec;44(12):3288–3297. PubMed PMID: 11083629; PubMed Central PMCID: PMC90194.
58. Pitt R, Alexander S, Ison C, et al. Phenotypic antimicrobial susceptibility testing of *Chlamydia trachomatis* isolates from patients with persistent or successfully treated infections. J Antimicrob Chemother. 2017 Nov 30. DOI:10.1093/jac/dkx454. PubMed PMID: 29207004.
59. Lau A, Kong F, Fairley CK, et al., Treatment efficacy of azithromycin 1 g single dose versus doxycycline 100 mg twice daily for 7 days for the treatment of rectal chlamydia among men who have sex with men - a double-blind randomised controlled trial protocol. Bmc Infect Dis. 2017 Jan 6;17(1):35. DOI:10.1186/s12879-016-2125-7. PubMed PMID: 28061753; PubMed Central PMCID: PMC5217553.
60. Shaw EI, Dooley CA, Fischer ER, et al. Three temporal classes of gene expression during the *Chlamydia trachomatis* developmental cycle. Mol Microbiol. 2000 Aug;37(4):913–925. PubMed PMID: 10972811.
• **This study showed that the chlamydia developmental cycle consists of three temporal classes of gene expression, identifying potential mRNA targets of interest.**
61. Belland RJ, Zhong G, Crane DD, et al. Genomic transcriptional profiling of the developmental cycle of *Chlamydia trachomatis*. Proc Natl Acad Sci USA. 2003 Jul 8;100(14):8478–8483. PubMed PMID: 12815105; PubMed Central PMCID: PMC166254.
62. Nicholson TL, Olinger L, Chong K, et al. Global stage-specific gene regulation during the developmental cycle of *Chlamydia trachomatis*. J Bacteriol. 2003 May;185(10):3179–3189. PubMed PMID: 12730178; PubMed Central PMCID: PMC154084.
63. Witkin SS, Minis E, Athanasiou A, et al. Chlamydia trachomatis: the persistent pathogen. Clin Vaccine Immunol. 2017 Oct;24(10). PubMed PMID: 28835360; PubMed Central PMCID: PMC5629669. DOI:10.1128/CVI.00203-17.
64. Hellyer TJ, DesJardin LE, Hehman GL, et al. Quantitative analysis of mRNA as a marker for viability of mycobacterium tuberculosis. J Clin Microbiol. 1999 Aug 13;37(2):290–295. /received 10/21/rev-request 10/28/accepted. PubMed PMID: 9889206; PubMed Central PMCID: PMC84288.
65. Rudi K, Moen B, Dromtorp SM, et al. Use of ethidium monoazide and PCR in combination for quantification of viable and dead cells in complex samples. Appl Environ Microbiol. 2005 Feb;71(2):1018–1024. PubMed PMID: 15691961; PubMed Central PMCID: PMC546808.
66. Nogva HK, Dromtorp SM, Nissen H, et al. Ethidium monoazide for DNA-based differentiation of viable and dead bacteria by 5'-nuclease PCR. Biotechniques. 2003 Apr;34(4):804–8, 810, 812–3. PubMed PMID: 12703305.
• **Key paper in the area of V-PCR methodology.**
67. Nocker A, Cheung CY, Camper AK. Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. J Microbiol Methods. 2006 Nov;67(2):310–320. PubMed PMID: 16753236.
68. Coffman GL, Gaubatz JW, Yielding KL, et al. Demonstration of specific high affinity binding sites in plasmid DNA by photoaffinity labeling with an ethidium analog. J Biol Chem. 1982 Nov 25;257(22):13205–13207. PubMed PMID: 6754713.
69. Rudi K, Naterstad K, Dromtorp SM, et al. Detection of viable and dead Listeria monocytogenes on gouda-like cheeses by real-time PCR. Lett Appl Microbiol. 2005;40(4):301–306. PubMed PMID: 15752222.
70. Wang S, Levin RE. Discrimination of viable Vibrio vulnificus cells from dead cells in real-time PCR. J Microbiol Methods. 2006 Jan;64(1):1–8. PubMed PMID: 15932774.
71. Hein I, Flekna G, Krassnig M, et al. Real-time PCR for the detection of Salmonella spp. in food: an alternative approach to a conventional PCR system suggested by the FOOD-PCR project. J Microbiol Methods. 2006 Sep;66(3):538–547. PubMed PMID: 16564585.
72. Rogers GB, Stressmann FA, Koller G, et al. Assessing the diagnostic importance of nonviable bacterial cells in respiratory infections. Diagn Microbiol Infect Dis. 2008 Oct;62(2):133–141. PubMed PMID: 18692341.
73. Elizaquivel P, Aznar R, Sanchez G. Recent developments in the use of viability dyes and quantitative PCR in the food microbiology field. J Appl Microbiol. 2014 Jan;116(1):1–13. PubMed PMID: 24119073.
74. Moreno L, Aznar R, Sanchez G. Application of viability PCR to discriminate the infectivity of hepatitis A virus in food samples. Int J Food Microbiol. 2015 May 18;201:1–6. PubMed PMID: 25720326.
75. Wang L, Li Y, Mustapha A. Detection of viable Escherichia coli O157:h7by ethidium monoazide real-time PCR. J Appl Microbiol. 2009 Nov;107(5):1719–1728. PubMed PMID: 19457030.
76. Delgado-Viscogliosi P, Solignac L, Delattre JM. Viability PCR, a culture-independent method for rapid and selective quantification of viable Legionella pneumophila cells in environmental water

samples. Appl Environ Microbiol. 2009 Jun;75(11):3502–3512. PubMed PMID: 19363080; PubMed Central PMCID: PMC2687295.

77. Inoue H, Fujimura R, Agata K, et al. Molecular characterization of viable Legionella spp. in cooling tower water samples by combined use of ethidium monoazide and PCR. Microbes Environ. 2015;30 (1):108–112. PubMed PMID: 25736979; PubMed Central PMCID: PMC4356457.
78. Yanez MA, Nocker A, Soria-Soria E, et al. Quantification of viable Legionella pneumophila cells using propidium monoazide combined with quantitative PCR. J Microbiol Methods. 2011 May;85 (2):124–130. PubMed PMID: 21329735.
79. Janssen KJ, Hoebe CJ, Dukers-Muijrers NH, et al. Viability-PCR shows that NAAT detects a high proportion of DNA from non-viable *Chlamydia trachomatis*. PLoS One. 2016;11(11):e0165920. PubMed PMID: 27812208; PubMed Central PMCID: PMC5094775.
    - **Study that reports on the validation of the V-PCR method to assess chlamydia viability in vaginal swab samples.**
80. Dukers-Muijrers NH, Wolffs PF, Eppings L, et al. Design of the FemCure study: prospective multicentre study on the transmission of genital and extra-genital *Chlamydia trachomatis* infections in women receiving routine care. BMC Infect Dis. 2016 Aug 8;16:381. PubMed PMID: 27502928; PubMed Central PMCID: PMC4977887.
81. Cangelosi GA, Meschke JS. Dead or alive: molecular assessment of microbial viability. Appl Environ Microbiol. 2014 Oct;80(19):5884–5891. PubMed PMID: 25038100; PubMed Central PMCID: PMC4178667.
82. van den Broek IVF, Land JA, van Bergen JEAM, et al. Chlamydia trachomatis antibody testing in vaginal mucosal material versus blood samples of women attending a fertility clinic and an STI clinic. Obstet Gynecol Int. 2014;2014:1–9. PubMed PMID: 24757446; PubMed Central PMCID: PMC3976833.
83. Yamamoto S, Azuma E, Muramatsu M, et al. Significance of extracellular vesicles: pathobiological roles in disease. Cell Struct Funct. 2016 Nov 25;41(2):137–143. PubMed PMID: 27679938.
84. Yamamoto S, Niida S, Azuma E, et al. Inflammation-induced endothelial cell-derived extracellular vesicles modulate the cellular status of pericytes. Sci Rep. 2015 Feb 17;5:8505. PubMed PMID: 25687367; PubMed Central PMCID: PMC4330530.