Journal of Virological Methods 203 (2014) 33–38

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Short communication

A simple and novel modification of comet assay for determination of bacteriophage mediated bacterial cell lysis

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Article history:
Received 5 December 2013
Received in revised form 13 February 2014
Accepted 21 February 2014
Available online 26 March 2014

Keywords:
Comet assay
In vitro toxicity
Virology research
Plaque assay
Bacteriophage

ABSTRACT

The comet assay is the widely used method for in vitro toxicity testing which is also an alternative to the use of animal models for in vivo testing. Since, its inception in 1984 by Ostling and Johansson, it is being modified frequently for a wide range of application. In spite of its wide applicability, unfortunately there is no report of its application in bacteriophages research. In this study, a novel application of comet assay for the detection of bacteriophage mediated bacterial cell lysis was described. The conventional methods in bacteriophage research for studying bacterial lysis by bacteriophages are plaque assay method. It is time consuming, laborious and costly. The lytic activity of bacteriophage devours the bacterial cell which results in the release of bacterial genomic material that gets detected by ethidium bromide staining method by the comet assay protocol. The objective of this study was to compare efficacy of comet assay with different assay used to study phage mediated bacterial lysis. The assay was performed on culture isolates ($N = 80$ studies), modified comet assay appear to have relatively higher sensitivity and specificity than other assay. The results of the study showed that the application of comet assay can be an economical, time saving and less laborious alternative to conventional plaque assay for the detection of bacteriophage mediated bacterial cell lysis.

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1. Introduction

The single cell gel electrophoresis assay, which is also known as “comet assay” is a sensitive technique for the detection of DNA damage and DNA repair quantitatively and qualitatively in single cells (Olive and Banath, 2006). This method was first developed by Ostling and Johanson, 1984 and later modified for use under alkaline conditions by Singh et al. in 1988 (Moller, 2006; Singh, 2000; Singh et al., 2003), producing a sensitive version of the assay which can assess double stranded DNA breaks, single stranded DNA breaks, detect alkali-labile sites expressed due to strand breaks in DNA and single-strand breaks associated with incomplete excision repair (Olive and Banath, 2006). The assay is also effective in detecting DNA–DNA and DNA–protein crosslinking. The detection of altered DNA migration is dependent on various parameters such as the concentration of agarose in the gel, the pH, temperature and duration of alkaline unwinding and the pH, temperature, voltage, amperage and duration of electrophoresis.

Comet assay has been standardized for evaluation of DNA damage/repair (Spivak, 2010; Singh, 2000; Singh et al., 2003), biomonitoring and genotoxicity testing (Speit and Hartmann, 1999, 2005). The term “comet” refers to the pattern of DNA migration through the electrophoresis gel, which often resembles a comet (Avishai et al., 2002). Electrophoresis at high pH results in structures resembling comets, observed by fluorescence microscopy; the intensity of the comet tail relative to the head reflects the number of DNA breaks. This is followed by visual analysis with staining of DNA and calculating fluorescence to determine the extent of DNA damage. This can be performed by manual scoring or automatically by imaging software (Avishai et al., 2002).

The assay is well established method and has gained wide acceptance as a valuable tool to analyze white blood cells or lymphocytes in human biomonitoring studies (Kassie et al., 2000) and various other cells (Szeto et al., 2005; Mussali-Galante et al., 2005; Emri et al., 2004; Graham-Evans et al., 2004; Rojas et al., 2000; Augustowska et al., 2007; Fraser, 2004; Schmid et al., 2007; Speit and Hartmann, 1999) with the simultaneous detection of single and double stranded breaks (Enciso et al., 2009). It makes feasible the studies related to the fundamental DNA damage and repair

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http://dx.doi.org/10.1016/j.jviromet.2014.02.028
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(Speit and Hartmann, 2005) and routine genotoxicity testing and assessment of industrial chemicals, biocides, agrochemicals and pharmaceuticals (Moller, 2005; Kirkland, 1990).

The assay has been modified by various workers at various steps, including lysis and electrophoretic conditions to make it suitable for assessing different types of DNA damage in different cell types (Hartmann and Speit, 1997; Hartmann et al., 2001; Collins, 2004; Hovhannisyan, 2010; Speit and Hartmann, 2005). Also, many researchers have already reported and have reviewed comet assay method with modification for wide applications (Fairbairn et al., 1995, 1996; Anderson et al., 1998; Rojas et al., 1999; Speit and Hartmann, 2005). We attempt to investigate the capacity of a modified comet assay to analyze phage mediated bacterial lytic activity. To the best of our knowledge this is the first application of a comet assay for studying the phage mediated bacterial lytic activity.

However, there are other reported methods for detecting phage mediated bacterial lysis like plaque assay (Adams, 1959), spot assay (Chopin et al., 1976) and streak assay (Merabishvili et al., 2009). In this study, the efficacy of various methods like spot assay, streak assay and modified comet assay was elucidated by comparing with existing gold standard plaque assay. These tests are time-consuming and tedious. Comet assay is comparatively more rapid than conventional methods.

2. Materials and methods

2.1. Bacterial strains

For the present study, different bacterial host strains isolated from environmental sources, i.e., wastewater, soil sample, lake water, etc. since last two years was selected (Table 1). The other bacterial strains were obtained from the American Type Culture Collection, USA and also from the National Collection of Type Cultures (NCTC), UK. The strains were maintained in tryptone soya agar (TSA) agar medium, supplemented with 3% NaCl. For long-term preservation, bacteria were frozen in tryptone soy broth (TSB) supplemented with 1% NaCl and 25% glycerol. Bacterial cultures were routinely prepared in tryptone soy broth (TSB; Himedia) and incubated at proper growth condition.

2.2. Isolation of bacteriophage

Bacteriophages were isolated from different environmental sample collected around Nagpur, India following enrichment procedure. Samples were first treated with chloroform and centrifuge at $5000 \times g$ to allow larger suspended solid to settle out. This sample was then passed through 0.22-μm pore-size filter (Millipex GP, Millipore, Cork, Ireland). Lytic bacteriophages were enriched selectively by mixing filtered sample with double strength TSB inoculated with bacterial host cultures incubated overnight at proper growth conditions. After incubation chloroform was added and the sample was stored at 4 °C. These samples were assayed for the phage by using different host cultures.

2.3. Methods to study phage mediated bacterial lysis

The study was conducted according to a standard method recommended for bacteriophage assay. The outline of various reported methods for detecting phage mediated bacterial lysis has been described below. The method generally used to assay phage is plaque assay method (Adams, 1959). In this study, plaque assay is a gold standard for comparing the modified comet assay method and different method. The result focused on the sensitivity and specificity of various methods used to study phage mediated bacterial lysis. In order to check the sensitivity and specificity of the modified comet assay, different methods for phage mediated bacterial lysis was compared with standard plaque assay method as described below.

2.3.1. Plaque assay – a standard test method for comparison with the other assay test method

The plaque assay requires the use of a Double-Layer Agar (DLA) technique also known as a double agar overlay method. In brief, the hard agar serves as a base layer and a mixture of few phage particles (10^7 PFU/ml) and host cells (1×10^9 CFU/ml) in a soft agar forms the upper overlay. Then the plates were incubated at 37° c for 24 h, susceptible bacterial cells multiply rapidly and produce a lawn of confluent growth in the medium. When one phage particle adsorbs to a susceptible cell, penetrates the cell, replicates and release new phage particles which infect other bacteria in the vicinity of the initial host cell. The growth or spread of the new viruses is then restricted or limited to the neighboring cells by the gel. The destroyed cells produce single circular, non-turbid areas called plaques in the bacterial lawn, where there is no growth of bacteria because the phage progeny originating from single virus particles have multiplied sufficiently to kill bacteria over an easily visible area.

2.3.2. Streak test assay

The streak test was used to test the presence of lytic phage (Merabishvili et al., 2009). In brief, a high titer phage suspension (10^7 PFU/ml) was dragged down the middle of a TSA agar plate with the help of sterile loop. Add bacterial host culture or bacterial suspension (1×10^9 PFU/ml) streak perpendicular to the phage streak, going all the way across in one movement, not going both ways. Allow it to air dried at room temperature for 15 min in biosafety cabinet. Plates were incubated at 37 °C for 24 h and lysis were observed. This method is a little harder to interpret sometimes, but it is a fairly easy way to pick out the most sensitive strains of bacteria.

2.3.3. Spot assay

The spot test method was used for the presence of phage by measuring phage lytic activity (Chopin et al., 1976). In brief, approximately 0.1 ml of mid-log phase host bacterial culture (1×10^9 CFU/mL) was flood onto a plate containing tryptone soya agar (TSA) and allowed to dry in a laminar flow biosafety cabinet. Ten microliters of phage sample were then dropped onto the plate containing lawn of host bacterial and incubated at 37 °C. A clear zone in the plate due to the lysis of host cells indicated the presence of lytic phage.

2.3.4. Modified comet assay

2.3.4.1. Preparation of slide. 200 μl of 1% agarose was added at both ends of the slide and cover slips placed over the agarose. After solidification, cover slips was removed. A solution of *Escherichia coli* (ATCC 13706) and coliphage (ATCC 13706 B1) (approximately 20 μl each) in 2 ml eppendorff tube vial was a test sample and the control sample without coliphage (ATCC 13706 B1) in 2 ml Eppendorf tube was prepared. Low melting agarose (1%) was added in the Eppendorf tube to make it 200 μl. Mixture of control and test samples was added at different ends of the agarose coated slide and cover slips placed over it. Allow it to solidify (∼5–10 min). Cover slip was slide off and finally a third layer of 1% agarose (200 μl) added over the agarose encapsulated control and test samples. Place cover slips over it, allow it to solidify (∼5–10 min). Slide incubated for 4–6 h at 37° C in an incubator (Singh et al., 1988). Lysis treatment is not required for this method as prokaryotic cells are lysed by their respective phages through their lytic activity. The detailed procedure is given in the flowchart (Fig. 1).

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