A modified MS2 bacteriophage plaque reduction assay for the rapid screening of antiviral plant extracts

Ian Cock, F. R. Kalt[1]

Biomolecular and Physical Sciences, Nathan Campus, Griffith University, 170 Kessels Road, Nathan, Queensland 4111, [1]Genomics Research Center, Gold Coast Campus, Griffith University, Parklands Drive, Southport, Queensland 4222, Australia

Submitted: 05-01-2010 Revised: 24-01-2010 Published: 07-09-2010

ABSTRACT

Introduction: Traditional methods of screening plant extracts and purified components for antiviral activity require up to a week to perform, prompting the need to develop more rapid quantitative methods to measure the ability of plant based preparations to block viral replication. We describe an adaption of an MS2 plaque reduction assay for use in *S. aureus*. **Results:** MS2 bacteriophage was capable of infecting and replicating in *B. cereus*, *S. aureus* and F+ *E. coli* but not F- *E. coli*. Indeed, both *B. cereus* and *S. aureus* were more sensitive to MS2 induced lysis than F+ *E. coli*. When MS2 bacteriophage was mixed with *Camellia* sinensis extract (1 mg/ml), *Scaevola spinescens* extract (1 mg/ml) or *Aloe barbadensis* juice and the mixtures inoculated into *S. aureus*, the formation of plaques was reduced to 8.9 ± 3.8%, 5.4 ± 2.4% and 72.7 ± 20.9% of the untreated MS2 control values respectively. **Conclusions:** The ability of the MS2 plaque reduction assay to detect antiviral activity in these known antiviral plant preparations indicates its suitability as an antiviral screening tool. An advantage of this assay compared with traditionally used cytopathic effect reduction assays and replicon based assays is the more rapid acquisition of results. Antiviral activity was detected within 24 h of the start of testing. The MS2 assay is also inexpensive and non-pathogenic to humans making it ideal for initial screening studies or as a simulant for pathogenic viruses.

Key words: Aloe vera, antiviral assay, *Camellia sinensis*, MS2 bacteriophage, plaque reduction assay, *Scaevola spinescens*

INTRODUCTION

The market for antiviral drugs is estimated at approximately US$ 20 billion per year and this figure is expected to increase as new antiviral agents become available. Following the recent worldwide H1N1 influenza outbreak, sales of antiviral drugs (especially oseltamivir phosphate (Tamiflu)) are expected to vastly increase during the 2009 – 2010 period. In 1990, there were only five licensed antiviral drugs; today this figure has grown to over 60.[1] However, most of these drugs are targeted at human immunodeficiency virus (HIV) and various herpes viruses. There is an urgent need to develop therapies against the myriad of viral diseases for which no therapies currently exist. As most medically important human viruses are RNA viruses,[2] the discovery of agents directed against RNA viruses is particularly important.

Address for correspondence:
Dr. Ian Cock, Biomolecular and Biomedical Sciences, Griffith University, Nathan Campus, 170 Kessels Road, Nathan, Queensland 4111, Australia, E-mail: I.Cock@griffith.edu.au

DOI: 10.4103/0974-8490.69108

Discovery and development of effective antiviral agents is a difficult task and has had limited success. As viruses use host cells to replicate, finding targets for drugs that eliminate the virus without harming the host cells is vital. The plant kingdom contains many unique unclassified compounds that are yet to be screened for anti-viral properties and may provide drug candidates for the treatment of viral diseases. For these agents to be successful as anti-viral agents, limited toxicity toward human cells is necessary.

The development of safe, cheap, rapid, high throughput assays is essential for the discovery of antiviral drugs from plants. Currently, most assays are based on virus-induced cytopathic effect reduction assays (CPE-RA)[3-5] or genomic/subgenomic replicon-based assays.[6-8] Although the CPE-RA and replicon-based assays are specific for viruses and cell lines, they are expensive, time consuming, and require specialized equipment and training. Both these methods involve growing cell lines for several days, inoculating them with the test virus, treating them with the potential antiviral agent, and after several more days, checking for a response (eg. reduction of the cytopathic effect or inhibition of viral replication). The time required for these assays and/or their low throughput nature limit

their use for initial screening of large sample groupings. Instead, CPE-RA and genomic/subgenomic replicon-based assays are more suited for directed antiviral analysis.

Thus, there is a need to develop alternative preliminary screening methods to be used prior to the cell screening assays. These methods should be more rapid, cheap, have equal or greater sensitivity, provide high levels of efficiency, and be safe for use in the laboratory. For these reasons MS2 bacteriophage was chosen for the development of a plaque reduction bioassay as an initial anti-viral screening tool.

MS2 bacteriophage (family Leviviridae) is a small (27 – 34 nm) icosahedral bacteriophage, which is usually described as an F pilin/male-specific bacteriophage of *Escherichia coli*.[9] Indeed, no evidence was found in the literature of MS2, infecting bacteria other than F+ *E. coli*. The MS2 genome consists of a single sense (+) RNA strand, 3569 nucleotides long, which contains four genes [Figure 1]. These encode proteins necessary for phage maturation, encapsidation, lysis of the bacterial host, and bacteriophage RNA replication.[10,11]

MS2 bacteriophage is an attractive virus for the development of an assay to screen for viral inactivation as most medically important human viruses are RNA viruses.[2] MS2 phage resembles the structure and function of many human enteroviruses (polioviruses 1-3, coxsakieviruses, echoviruses, and enteroviruses 68-72)[12] making it a relevant model system. It is readily cultivatable in titres up to 10^{12} pfu/ml and enumeration is rapid (less than 24 hours), and it is inexpensive. Furthermore, MS2 in not pathogenic to humans and can therefore be tested in high numbers without the need for additional safety measures. This makes MS2 bacteriophage a useful simulant in place of small, human infective RNA viruses (eg., Ebola virus, Marburg virus, and equine encephalitis alphaviruses).

The current studies were undertaken to adapt an MS2 plaque reduction assay that is routinely used to test environmental water quality[12] and to optimize the assay for routine screening of plant extracts. To test this assay we chose to examine plants that have previously been shown to inhibit viral growth to determine whether antiviral activity could also be detected in the plaque reduction assay. Recent studies have shown that *Camellia sinensis* (tea) inhibits influenza virus replication in the Madin-Darby canine kidney (MDCK) cell line,[13] blocks attachment of HIV in T cells,[14] and has been reported to be effective in the treatment of human papillomavirus (HPV)-induced genital warts.[15] A previous study[16] found *Scaevola spinescens* leaves to be capable of inhibiting greater than 25% of human cytomegalovirus (CMV) production. Similarly, *Aloe barbadensis* Miller extracts have also been shown to inhibit CMV production in human cell lines.[17] Furthermore, aloe emodin, purified from *A. barbadensis,* has been shown to inactivate herpes simplex virus type 1 and type 2, the varicella-zoster virus, pseudorabies virus, and influenza virus.[18] In this report, we outline the use of the MS2 plaque reduction assay to detect antiviral activity in *C. sinensis*, *S. spinescens* extracts, and *A. barbadensis* juice.

MATERIALS AND METHODS

Viral and Bacterial Stocks

MS2 bacteriophage, F+, and F- Amp+ *E. coli* used in this study were supplied by Dr. Jatinder Sidhu and Dr. Simon Toze of CSIRO, St. Lucia Qld, Australia. *Bacillus cereus* and *Staphylococcus aureus* were obtained from Michelle Mendell and Tarita Morais, Griffith University. All stock cultures were subcultured and maintained in nutrient broth at 4°C.

Production of MS2 virus

One hundred milliliters of nutrient broth (25 g/l) containing ampicillin (100 µg/ml) was inoculated with either 1 ml F+ Amp+ *E. coli* culture or 1 ml of F- Amp+ *E. coli* culture and incubated overnight at 37°C. Parallel studies examined the ability of *B. cereus* and *S. aureus* to

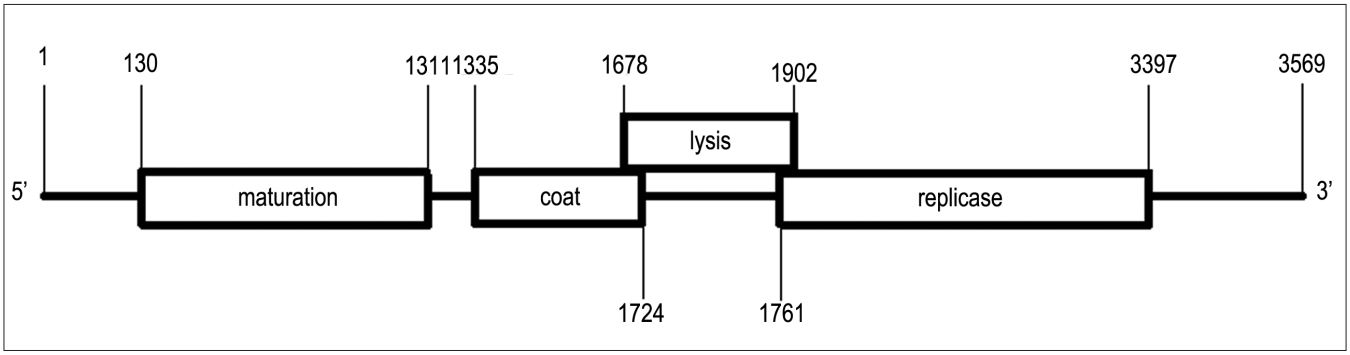

Figure 1: Genetic map of the MS2 bacteriophage. Nucleotide positions for the start and end of each gene are noted in the figure. With regard to the overlapping genes, the coat and replicase genes are read in the same frame, whereas, the lysis gene reading frame is +1 with respect to these genes.

produce MS2 bacteriophage. One milliliter of *B. cereus* or *S. aureus* were inoculated into 100 ml of nutrient broth (25 g/l) and incubated overnight at 37°C. The following day, flasks containing 30 ml of nutrient broth (containing 100 µg/ml ampicillin for *E. coli* cultures or without ampicillin for *B. cereus* and *S. aureus* cultures) were inoculated with 1 ml of the relevant culture and incubated for two hours at 37°C and 160 rpm. Once the bacterial cells had reached log phase, 1 ml of stock MS2 virus (containing approximately 10^8 plaque forming units) was added and incubated overnight at 35°C. The solution was centrifuged at 4000 rpm for 10 minutes and the supernatant was collected and passed through a 22 µm Sarstedt filter. All stock and working solutions were stored at 4°C until further use.

Determination of MS2 virus cDNA Synthesis

cDNA synthesis was carried out using an iScript Select cDNA Synthesis Kit, (Bio-Rad Laboratories, Inc., USA) as per the manual instructions. Briefly, 1 µl reverse transcriptase, 4 µl 5 x iScript Select reaction mix, 1 µl random primers (hexamers), and 13 µl RNA samples were added to the individual PCR tubes. A Biorad C1000 thermocycler reaction program employing the following steps was used: Five minutes at 25ºC for primer annealing, 30 minutes at 42ºC for cDNA synthesis, and a final incubation step of five minutes at 85ºC to deactivate the reverse transcriptase.

cDNA Polymerase Chain Reaction Amplification

Polymerase chain reaction (PCR) using an Invitrogen PCR SuperMix was performed using the synthesized cDNA as a template. Briefly, 10 µl Master mix, 1 µl primer mix containing 0.5 µl of forward primer (MS2-109 CAT AGG TCA AAC CTC CTA GGA ATG), 0.5 µl reverse primer (MS2-21 TCC TGC TCA ACT TCC TGT CGA G), and 9 µl of each cDNA preparation were added to the reaction tubes. PCR was performed using a Biorad C1000 thermocycler comprising of a denaturing step (95ºC, 30 seconds) annealing step (58ºC, 30 seconds), and extension step (72ºC, 30 seconds) for 32 cycles, and a final extension step of 72ºC for five minutes followed by a cooling step of 4ºC for 15 minutes.

Agarose Gel Electrophoresis

The PCR products were run on 3% Agarose gel against a positive control (fresh MS2 virus) in order to determine whether the MS2 bacteriophage was produced by each of the bacterial species tested.

Plant Test Samples

A. barbadensis juice was obtained from Aloe Wellness Pty Ltd., Australia, and was stored at 4ºC until use. *C. sinensis* leaf extract was obtained by immersing a single tea bag (Lipton) in 50 ml deionized water for four hours at room temperature, with constant mixing. *S. spinescens* plant material was provided by Jeannie Cargo of Outback Books (an online supplier of *S. spinescens* tea) as pre-dried and coarse milled whole plant material. One gram of plant material was extracted in deionized water for four hours at room temperature with constant mixing. Following extraction, the liquid was filtered using Whatman No. 54 filter paper, followed by rotary evaporation in an Eppendorf concentrator 5301. The resultant dry extract was weighed and redissolved in 10 ml deionized water.

Soft Agar Overlay

A soft agar overlay was prepared to a final concentration of 0.7% w/v Agar, 1% w/v Glucose, 1% w/v $CaCl_2$ solution, and 1% w/v $MgSO_4$, and autoclaved at 120°C for 20 minutes. The soft agar overlay was allowed to cool to 65°C, and then nalidixic acid was added to a final concentration of 0.4% w/v. The overlay was used immediately for the MS2 plaque inhibition assay described later in the text.

MS2 Plaque Inhibition Assay

Prior to plating, 490 µl of crude plant extract was inoculated with 10 µl of MS2 virus (containing approximately 10^{10} plaque forming units/ml) and incubated overnight at 4ºC. The solution was added to 500 µl *B. cereus*, *E. coli* or *S. aureus* as required and incubated at 37ºC for 20 minutes. The bacteria/virus/extract mixture was then added to 3 ml soft agar overlay and immediately poured over pre-made agar plates (2.8% w/v Agar). The plates were allowed to set for 15 minutes at room temperature, inverted, and incubated overnight at 37ºC. The following morning, the plaques were counted and the percentage inhibition recorded. Serial dilution was used to determine the antiviral strength of the samples where necessary. Nutrient broth and deionized water were used as negative controls, while *C. sinensis* extract, *S. spinescens* extract, *A. barbadensis* juice, and UV irradiation (microwave of 10 µl virus only for 4 × 30 seconds) were used as positive controls.

Statistical Analysis

Data are expressed as the mean ± SD of at least three independent experiments. The Paired *t*-test was used to calculate the statistical significance between the control and treated groups, with a P value < 0.05 considered to be statistically significant.

RESULTS

MS2 production and lysis of various bacteria

Early in this study, MS2 virus production was tested in F+ *E. coli*, F- *E. coli*, *B. cereus,* and *S. aureus*. Although no lysis was seen in F- *E. coli* (as seen by the lack of bacterial

cell debris in the culture) and limited lysis was seen in the F+ *E. coli*, both *B. cereus* and *S. aureus* were highly prone to bacteriophage-induced lysis (as seen by the degree of bacterial cell debris). This is an interesting finding as previous reports have described MS2 bacteriophage as being specific to F+ *E. coli*. MS2 production studies using the other bacterial species were initially included as negative controls. The lysis in the *B. cereus* and *S. aureus* cultures indicates that MS2 may not be as specific as previously reported. Moreover, increased lysis in the *B. cereus* and *S. aureus* cultures indicates that these bacteria are more sensitive to MS2 lysis than are F+ *E. coli*, and could therefore be used to develop a more sensitive bioassay than the current assay used to measure plaque reduction activity of water samples.

To provide evidence that the lysis seen in *B. cereus* and *S. aureus* cultures was due to MS2 bacteriophage infection/production, cDNA was produced against RNA from the cell-free culture media and this cDNA was amplified using primer sequences specific to the 5' non-coding region of the MS2 bacteriophage. As seen in Figure 2, culturing MS2 in F- *E. coli* (lane B) resulted in the production of no MS2 cDNA. In contrast, MS2 phage cDNA was clearly evident in the samples synthesized against the MS2/ F+ *E. coli* culture. Interestingly, cDNA synthesized against both the *S. aureus* (lane F) and *B. cereus* (lane G) MS2 cultures also clearly showed the presence of phage cDNA, indicating that these bacteria were indeed capable of MS2 production. It was therefore evident that MS2 bacteriophage is not F+ *E. coli* specific, as had been previously described, but could infect a wider array of bacterial species. Even as the levels of MS2 cDNA production appeared similar in F+ *E. coli*, *B. cereus,* and *S. aureus* in this study, the latter two bacterial species were more sensitive to lysis than was F+ *E. coli*.

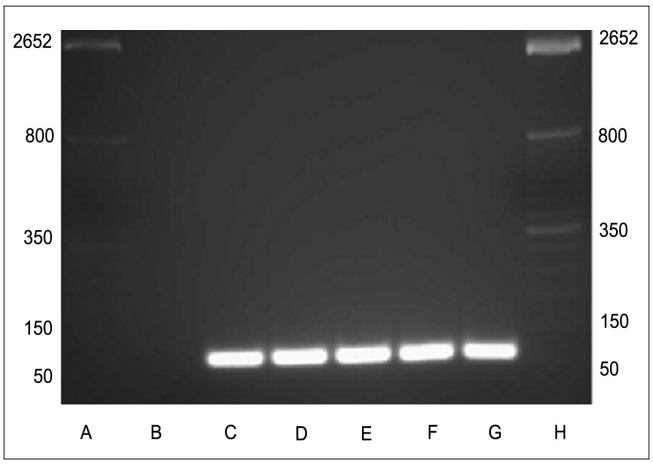

Figure 2: 3% Agarose gel of PCR products from cDNA synthesized against MS2 viral RNA. (A and H) DNA bp ladder, (B) MS2 bacteriophage produced in F- *E. coli*, (C and D) MS2 bacteriophage obtained from separate CSIRO stocks, (E) MS2 bacteriophage produced in F+ *E. coli*, (F) MS2 bacteriophage produced in *S. aureus*, and (G) MS2 bacteriophage produced in *B. cereus*. Tests were performed in triplicate and representative results are shown here.

To develop a more sensitive assay, the ability of the MS2 bacteriophage to produce plaques in F+ *E. coli*, *B. cereus,* and *S. aureus* was compared. Plaque production in F- *E. coli* was also determined as a negative control. Table 1 shows the number of plaques seen when 500 µl of undiluted MS2 (containing 10^{10} plaque forming units/ml) or 500 µl of a 1 in 50 dilution of MS2 (containing 2×10^8 plaque forming units/ml) were incubated with 500 µl of the bacterial stocks and plated. No plaque counts were available for the *B. cereus* assays as very little bacterial growth was seen in the presence of MS2 at the levels tested. It is likely that while MS2 production is effective in *B. cereus*, lysis of the bacteria is so complete that *B. cereus* is unsuitable for use as a test bacterium. Concentration-response testing of MS2 in *B. cereus* (unpublished results) showed an all or nothing response. As the levels of MS2 plaque forming units were further reduced to 5×10^7 plaque forming units/ml, the ability to inhibit bacterial growth was lost. However, at the same concentration, no plaques were seen.

MS2 was tested at the concentrations indicated. The number of plaques seen was recorded as the mean ± standard deviation of at least triplicate determinations.

In contrast, high plaque numbers were seen in both F+ *E. coli* and *S. aureus* when they were incubated with 5×10^9 plate forming units of MS2. Indeed, in both cases the number of plaques was too many to count and was recorded as > 200. When a 1 in 50 dilution of the MS2 stock (10^8 plate forming units) was used, the plaque numbers on F+ *E. coli* plates decreased dramatically to approximately 24 plaques. *S. aureus* was more sensitive to MS2-induced lysis. When tested with the addition of 10^8 plaque forming units of MS2, approximately 180 plaques were seen on the *S. aureus* plates. The higher sensitivity of *S. aureus* to MS2 bacteriophage allowed for the detection of lower levels of the phage, and hence a more sensitive assay. The potential antiviral agents were tested against *S. aureus* in all remaining plaque reduction assays. No plaques were seen when MS2 was tested against F- *E. coli*.

Table 1: MS2 plaque counts in the test bacterial species

Bacteria	MS2 Added	
	5×10^9 pfu	10^8 pfu
F+ *E. coli*	> 200	23.6 ± 5.6
F- *E. coli*	0	0
B. cereus	NBG*	NBG*
S. aureus	> 200	178.3 ± 30.7

*NBG = no bacterial growth observed.

Screening of plant extracts

A. barbadensis juice, *C. sinensis* extract (1 mg/ml), and *S. spinescens* extract (1 mg/ml) were tested for the ability to inhibit MS2 plaque formation in *S. aureus* [Figure 3]. Microwave irradiation (positive control) of MS2 bacteriophage [Figure 3f] completely destroyed the plaque forming potential of the phage. All plant extracts tested also reduced the plaque formation in *S. aureus*. Both *C. sinensis* leaf extract [Figure 3c] and *S. spinescens* extract [Figure 3d] were particularly effective at inhibiting MS2 plaque formation. *A. barbadensis* juice, while also able to partially inhibit the formation of MS2 plaques, was substantially less effective than the other plant extracts.

Figure 4 shows the plaque formation in the presence of plant extracts as a percentage of plaque formation in the negative controls. No difference was seen between the negative controls (nutrient broth or water added to the MS2 bacteriophage instead of juice/extract). All plant extracts produced a statistically significant decrease in MS2 plaque production with *C. sinensis* and *S. spinescens* extracts almost completely blocking the plaque formation at a 1 mg/ml concentration.

The plant extracts were further tested over a range of concentrations to determine the minimum concentration capable of inhibiting 100% of the plaque formation (PI_{100}) and the minimum concentration capable of inhibiting 50% of the plaque formation (PI_{50}) [Table 2]. PI_{100} values were not obtained for *A. barbadensis* juice or *S. spinescens*, as none of the tested concentrations of these extracts was found to inhibit 100% of the plaque formation, even when tested undiluted. In contrast, *C. sinensis* leaf extract was capable of totally blocking MS2 plaque formation at a concentration of approximately 19.6 mg/ml. PI_{50} values were obtained for both *C. sinensis* leaf extract (4.9 ± 1.6 mg/ml) and *S. spinescens* extract (7.9 ± 2.6 mg/ml). No PI_{50} was obtained for *A. barbadensis* juice, as even when it was undiluted, the plaque counts did not decrease to 50% of the control value.

All PI_{100} and PI_{50} values are expressed as mg/ml ± standard deviation. NPI denotes that $PI_{100/}PI_{50}$ has not been achieved. All values are the means of at least triplicate determinations.

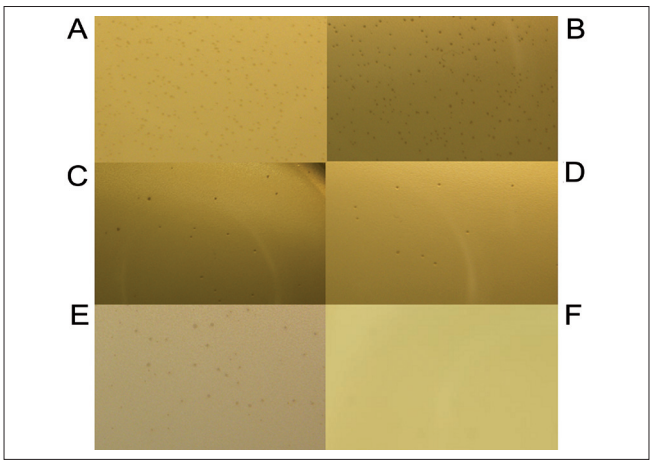

Figure 3: MS2 plaque reduction assay in an *S. aureus* lawn following incubation of the MS2 with (A) nutrient broth (negative control), (B) deionized water (negative control), (C) *C. sinensis* leaf aqueous extract 1 mg/ml), (D) *S. spinescens* water extract (1 mg/ml), (E) *A. barbadensis* juice, (F) nutrient agar, and microwave irradiation (positive control). All assays were performed in triplicate and representative assays are shown.

DISCUSSION

Understanding the mechanism of viral replication is not the only key step toward the identification of effective drugs against a virus. Development of rapid screening assays is also essential for antiviral drug discovery. The current study describes the development of an MS2 bacteriophage plaque reduction assay in *S. aureus*. The rapid nature of this test and its ease/low cost compared to other antiviral assay techniques makes it a valuable tool for rapidly screening potential antiviral agents, to target samples for more specific screens.

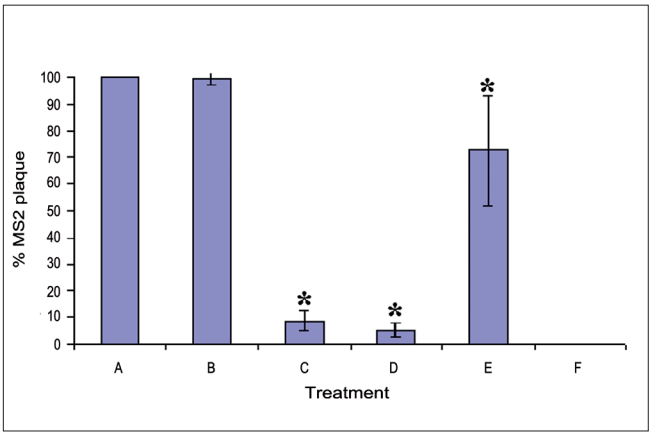

Figure 4: MS2 plaque formation presented as a percentage of untreated phage plaque formation following incubation of the MS2 phage with (A) nutrient media (negative control), (B) deionized water (negative control), (C) *C. sinensis* leaf water extract (1 mg/ml), (D) *S. spinescens* water extract (1 mg/ml), (E) *A. barbadensis* juice, (F) nutrient agar, and microwave irradiation (positive control). All results were reported as the mean of triplicate assays ± standard deviation. * indicates statistically significant results.

Table 2: Minimum concentrations capable of inhibiting 100% (PI_{100}) and 50% (PI_{50}) of the plaque formation for anti-viral plant samples

MS2 Phage Treatment	PI_{100}	PI_{50}
C. sinesis water extract	19.6 ± 6.5	4.9 ± 1.6
S. spinescens water extract	NPI	7.9 ± 2.6
A. barbadensis juice	NPI	NPI

These studies demonstrate that *S. aureus* is more sensitive to MS2-induced lysis than F+ *E. coli*. This was a surprising result as previous studies refer to MS2 as an F+ *E. coli*-specific bacteriophage. No studies were found in literature that discussed MS2 conjugation or lysis of other bacterial species. It has been demonstrated that MS2 phage uptake into *E. coli* cells is mediated through binding to the F pilin protein in the F pili.[19] Only F+ (but not F-) *E. coli* cells can take up MS2 bacteriophage, resulting in lysis. Indeed, F- *E. coli* cells were used in our studies as a negative control. Presumably any bacteria with F pilin expressed in their cell walls would be able to take up MS2. F+ plasmids can be easily transferred from *E. coli* cell to *E. coli* cell by conjugation.[19] It is also likely that a similar exchange is possible between different bacterial species. It is therefore surprising that there are no other reports of MS2 plaque formation in other bacterial species.

The formation of pili on the surface of Gram-negative bacteria has been studied in detail. In contrast, the pilus assembly pathways in Gram-positive bacteria have yet to be fully characterized. Gram-positive bacteria use cell wall peptidoglycan as a surface organelle for the covalent attachment of proteins.[20] This strategy involves sorting signals of surface protein precursors and sortase (a transpeptidase that cleaves sorting signals and links the C-terminus of surface proteins via an amide bond to the peptidoglycan cross-bridge).[20] To further validate the potential of *S. aureus* as a suitable host for the MS2 bacteriophage, it needs to be shown that *S. aureus* contains these mechanisms and possesses the ability to form pili. Related bacterial species (*Streptococcus pyogenes* and *Streptococcus pneumoniae*) have already demonstrated the ability to form pili.[21-23] Vegetative forms of *B. cereus* have also been reported to form pili.[24]

The sortase enzyme (Sortase A), a housekeeping enzyme, responsible for catalyzing cell-wall anchoring of surface proteins was discovered in *S. aureus* and is present in all Gram-positive bacterial genomes except in *Mycobacterium* and *Microplasma*.[25] Many pathogens harbor additional sortases, which are involved in iron acquisition, sporulation, and pilus assembly.[26] Sortases of the class C family form the largest group and are often present in multiple copies in a genome. These sortases are encoded together with their substrates, which constitute various types of pili in many pathogens.

S. aureus has been shown to possess two classes of sortases, Class A and Class D.[26,27] *B. cereus* has been shown to contain all four sortase subgroups.[27] As these sortases are responsible for the formation of pili on the surface of Gram-positive bacteria, this may explain the efficiency that the MS2 virus has in relation to *B. cereus* and *S. aureus*. Further tests examining the interaction of other sortase containing bacteria with the MS2 virus are necessary to provide an insight into bacterial lysis, sortase subfamily, and MS2 selectivity.

Although this assay was developed to test plant extracts for antiviral activity, a similar assay utilizing *E. coli* is routinely used to test environmental water quality.[12] The current study has demonstrated greater MS2 sensitivity in *S. aureus* compared to *E. coli*. Therefore, the use of *E. coli* as an enumeration tool for quantifying bacteriophage levels in environmental water samples may contribute to an understatement of contamination (levels of bacteriophages present).

As the common use of broad spectrum antibiotics, harsh chemicals, and irradiation has resulted in the emergence of highly resistant bacterial strains, new antibacterial treatments are always necessary. The presence of pili on bacterial cell surfaces and their demonstrated role in bacterial adherence make them ideal candidates for vaccines. As the MS2 virus is F pilin selective, an effective antibacterial treatment based on this selectivity could be developed. Currently, phage therapy (viruses that specifically target pathogenic bacteria) for humans is available only at the Phage Therapy Center in the Republic of Georgia and in Poland.[28] These institutes have used phage therapy to treat over 1500 patients with bacterial infections, when antibiotic treatment had failed.[28] This treatment is safe, highly effective, and may be applied to all patients from whom isolated bacterial strains have shown sensitivity to specific phages. Phage therapy has been used to prevent diarrhea caused by *E. coli*[28] and is of particular importance to the two pathogens: *S. aureus* and *Pseudomonas aeruginosa*, which have shown sensitivity to specific phages in more than 80% of the cases.[28]

Studies have already been conducted using the bacteriophage MSa, testing its activity against *S. aureus* in mice.[29] Following simultaneous inoculation with both MSa and lethal or non-lethal doses of *S. aureus,* MSa rescued 97% of mice from death and all non-lethal doses were fully resolved.[29] MSa phage can also prevent abscess formation and reduce the bacterial load and weight of abscesses. This suggests a potential use of the phage for the control of both local and systemic human *S. aureus* infections.[29]

These successful treatments will fuel a growing interest in the use of bacteriophages in medical and commercial practice. This is already evident. As of January 2, 2007, the United States FDA gave Omnilytics approval to apply its *E. coli* O157:H7 killing phage in a mist, spray or wash on live animals that will be slaughtered for human consumption.[30]

Although the current experiments yielded interesting

results with respect to the MS2 bacteriophage and its efficacy toward *S. aureus,* the ultimate goal is to develop and successfully conduct a trial of an anti-viral assay that is cheap, safe, simple, and allows for high throughput. Studies have shown that *A. barbadensis*, *C. sinensis,* and *S. spinescens* extracts inhibit viral growth in human cell lines.[16,31] *S. spinescens* and *C. sinensis* extracts have also shown significant inhibition against the MS2 bacteriophage in our plaque reduction assay. Although inhibition by *A. barbadensis* is not as pronounced in the current study, it is still evident when using this assay. However, it should be noted that comparing results seen against bacteriophage and viruses in human cell lines is ambiguous and future investigations using virally infected human cells lines are needed. By conducting these studies it will be possible to determine whether or not a direct comparison between this bacteriophage plaque assay and assays using viruses in human cell lines exists. If a correlation is seen, using this MS2 bacteriophage plaque assay as a first step for determining possible anti viral plants will be a valuable tool.

ACKNOWLEDGMENTS

The MS2 bacteriophage and the F+ and F- Amp+ *Escherichia coli* used in these studies were supplied by Dr. Jatinder Sidhu and Dr. Simon Toze of CSIRO, St Lucia Qld, Australia. The authors thank Dr. Sidhu and Dr. Toze of CSIRO and Ben Matthews of Griffith University for the advice and technical assistance given for the development of this assay for testing plant extracts. Financial support for this study was provided by the School of Biomolecular and Physical Sciences, Griffith University.

REFERENCES

1. De Clercq E. Highlights in the discovery of antiviral drugs: A personal retrospective. J Med Chem 2010;53:1438-50. online pre-publication manuscript: [accessed 2009 Nov 26].
2. Domingo E, Holland JJ. RNA virus mutations and fitness for survival. Annu Rev Microbiol 1997;51:151-78.
3. Li Q, Maddox C, Rasmussen L, Hobrath JV, White LE. Assay development and high-throughput antiviral drug screening against Bluetongue virus. Antiviral Res 2009;83:276-73.
4. Wan WB, Beadle JR, Hartline C, Kern ER, Ciesla SL, Valiaeva N, *et al*. Comparison of the antiviral activities of alkoxyalkyl and alkyl esters of cidofovir against human and murine cytomegalovirus replication *in vitro*. Antimicrob Agents Chemother 2005;49:656-62.
5. Kern ER, Hartline C, Harden E, Keith K, Rodriguez N, Beadle JR, *et al*. Enhanced inhibition of orthopoxvirus replication *in vitro* by alkoxyalkyl esters of cidofovir and cyclic cidofovir. Antimicrob Agents Chemother 2002;46:991-5.
6. Severson WE, McDowell M, Ananthan S, Chung DH, Rasmussen L, Sosa MI, *et al*. High-throughput screening of a 100,000-compound library for inhibitors of influenza A virus (H3N2). J Biomol Screen 2008;13:879-87.
7. Severson WE, Shindo N, Sosa M, Fletcher T 3rd, White EL, Ananthan S, *et al*. Development and validation of a high-throughput screen for inhibitors of SARS CoV and its application in screening of a 100,000-compound library. J Biomol Screen 2007;12:33-40.
8. Puig-Basagoiti F, Deas TS, Ren P, Tilgner M, Ferguson DM, Shi PY. High-throughput assays using a luciferase-expressing replicon, virus-like particles, and full-length virus for West Nile virus drug discovery. Antimicrob Agents Chemother 2005;49:4980-8.
9. Bollback JP, Huelsenbeck JP. Phylogeny, genome evolution, and host specificity of single-stranded RNA bacteriophage (family Leviviridae). J Mol Evol 2001;52:117-28.
10. Vinjé J, Oudejans SJ, Stewart JR, Sobsey MD, Long SC. Molecular detection and genotyping of male-specific coliphages by reverse transcription-PCR and reverse line blot hybridization. Appl Environ Microbiol 2004;70:5996-6004.
11. Groeneveld H, Thimon K, van Duin J. Translational control of maturation-protein synthesis in phage MS2: A role for the kinetics of RNA folding? RNA 1995;1:79-88.
12. Lazarova V, Savoys P. Technical and sanitary aspects of wastewater disinfection by UV irradiation for landscape irrigation. Water Sci Technol 2004;50:203-9.
13. Song JM, Lee KH, Seong BL. Antiviral effect of catechins in green tea on influenza virus. Antiviral Res 2005;68:66-74.
14. Nance CL, Siwak EB, Shearer WT. Preclinical development of the green tea catechin, epigallocatechin gallate, as an HIV-1 therapy. J Allergy Clin Immunol 2009;123:459-65.
15. Meltzer SM, Monk BJ, Tewari KS. Green tea catechins for treatment of external genital warts. Am J Obstet Gynecol 2009;200:233.e1-7.
16. Semple SJ, Reynolds GD, O'Leary MC, Flower RL. Screening of Australian medicinal plants for antiviral activity. J Ethnopharmacol 1998;60:163-72.
17. Saoo K, Miki H, Ohmori M, Winters WD. Antiviral Activity of Aloe Extracts against Cytomegalovirus. Phytotherapy Res 1996;10:348-50.
18. Sydiskis RJ, Owen DG, Lohr JL, Rosler KH, Blomster RN. Inactivation of enveloped viruses by anthraquinones extracted from plants. Antimicrob Agents Chemother 1991;35:2463-6.
19. Pererva TP, Miriuta Alu, Miriuta NIu. Interaction of RNA-containing bacteriophages with the host cells: MS2-induced *E. coli* mutants and formation of DNA-containing derivatives of MS2 bacteriophage. Tsitol Genet 2008;42:73-90.
20. Ton-That H, Schneewind O. Assembly of pili in Gram-positive bacteria. Trends Microbiol 2004;12:228-34.
21. Mora M, Bensi G, Capo S, Falugi F, Zingaretti C, Manetti AG, *et al*. Group A Streptococcus produce pilus-like structures containing protective antigens and Lancefield T antigens. Proc Natl Acad Sci U S A 2005;102:15641-6.
22. Barocchi MA, Ries J, Zogaj X, Hemsley C, Albiger B, Kanth A, *et al*. A pneumococcal pilus influences virulence and host inflammatory responses. Proc Natl Acad Sci USA 2006;103:2857-62.
23. LeMieux J, Hava DL, Basset A, Camilli A. RrgA and RrgB are components of a multisubunit pilus encoded by the *Streptococcus pneumoniae* rlrA pathogenicity islet. Infect Immun 2006;74:2453-6.
24. Budzik JM, Marraffini LA, Schneewind O. Assembly of pili on the surface of *Bacillus cereus* vegetative cells. Mol Microbiol 2007;66:495-510.
25. Shtatland T, Gill SC, Javornik BE, Johansson HE, Singer BS, Uhlenbeck OC, *et al*. Interactions of *Escherichia coli* RNA with bacteriophage MS2 coat protein: Genomic SELEX. Nucleic Acids Res 2000;28:E93.
26. Dramsi S, Trieu-Cuot P, Bierne H. Sorting sortases: A

nomenclature proposal for the various sortases of Gram-positive bacteria. Res Microbiol 2005;156:289-97.

27. Mandlik A, Swierczynski A, Das A, Ton-That H. Pili in Gram-positive bacteria: Assembly, involvement in colonization and biofilm development. Trends Microbiol 2008;16:33-40.
28. Sciences PAO. Therapeutic use of bacteriophages in bacterial infections. 2002; Available from: http://www.iitd.pan.wroc.pl/phages/phages.html. [accessed on 2009 Nov 26].
29. Capparelli R, Parlato M, Borriello G, Salvatore P, Iannelli D. Experimental phage therapy against *staphylococcus aureus* in mice. Antimicrob Agents Chemother 2007;51:2765-73.
30. Walbeck A. (2007). OmniLytics Announces USDA/FSIS Approval for Bacteriophage Treatment of *E. coli* O157:H7 on Livestock. 2007; Available from: http://www.businesswire.com/portal/site/google/index.jsp?ndmViewId=news_viewandnewsId=20070102005459andnewsLang=en. [accessed on 2009 Nov 26].
31. Ho HY, Cheng ML, Weng SF, Leu YL, Chiu DT. Antiviral effect of epigallocatechin gallate on enterovirus 71. J Agric Food Chem 2009;57:6140-7.

Source of Support: School of Biomolecular and Physical Sciences, Griffith University. **Conflict of Interest:** None declared.