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Identification of the inactivating factors and mechanisms exerted on MS2 coliphage in concentrated synthetic urine

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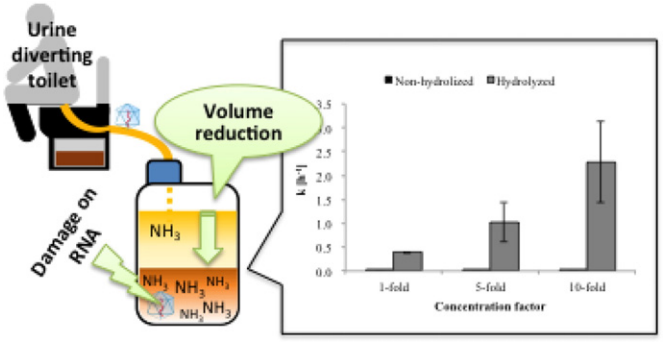
HIGHLIGHTS

- Uncharged ammonia is the predominant factor for MS2 inactivation in synthetic urine.
- Genome damage is the main mechanism for MS2 infectivity loss in synthetic urine.
- RT-qPCR targeting six genome regions can be used to monitor MS2 infectivity loss.
- MS2 infectivity loss in urine can be predicted by ion composition and speciation.

GRAPHICAL ABSTRACT



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ABSTRACT

Volume reduction (condensation) is a key for the practical usage of human urine as a fertilizer because it enables the saving of storage space and the reduction of transportation cost. However, concentrated urine may carry infectious disease risks resulting from human pathogens frequently present in excreta, though the survival of pathogens in concentrated urine is not well understood. In this study, the inactivation of MS2 coliphage, a surrogate for single-stranded RNA human enteric viruses, in concentrated synthetic urine was investigated. The infectious titer reduction of MS2 coliphage in synthetic urine samples was measured by plaque assay, and the reduction of genome copy number was monitored by reverse transcription-quantitative PCR (RTqPCR). Among chemical-physical conditions such as pH and osmotic pressure, uncharged ammonia was shown to be the predominant factor responsible for MS2 inactivation, independently of urine concentration level. The reduction rate of the viral genome number varied among genome regions, but the comprehensive reduction rate of six genome regions was well correlated with that of the infectious titer of MS2 coliphage. This indicates that genome degradation is the main mechanism driving loss of infectivity, and that RT-qPCR targeting the six genome regions can be used as a culture-independent assay for monitoring infectivity loss of the coliphage in urine. MS2 inactivation rate constants were well predicted by a model using ion composition and speciation in synthetic urine samples, which suggests that MS2 infectivity loss can be estimated solely based on the solution composition, temperature and pH, without explicitly accounting for effects of osmotic pressure.

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1. Introduction

Human urine is rich in nitrogen, phosphate, and potassium, which are essential components for food production. Average amounts of these nutrients (phosphate, nitrogen, and potassium) in human urine are approximately 5.6 kg, 0.5 kg, and 1.0 kg per person per year, respectively, and account for 37%, 19%, and 54% of global consumption of fertilizer (Wolgast, 1995), respectively. However, human urine is commonly mixed with feces, which may pose infectious disease risks and impede safe use of human urine as a fertilizer (Karak and Bhattacharyya, 2011). The introduction of a urine-diverting toilet is an effective approach for resource recovery from human excreta, but cross-contamination occurs (Schönning et al., 2002). A number of human pathogens, both viral and bacterial, could be detected in human urine collected from urine-diverting dry toilets (Bischel et al., 2015). The World Health Organization (WHO) reported that the storage of urine for longer than 6 months at temperatures higher than 20 °C in a closed tank achieved sufficient pathogen inactivation in urine in a large-scale system (WHO, 2006), but such long-term storage (more than a few months) may not be feasible for urine collection at a scale in dense urban environments due to storage volume requirements. The odor problem and the loss of recoverable nutrients by ammonia volatilization during long-term urine storage also have to be addressed.

Several treatment processes are available to tackle the limitations of urine storage (Maurer et al., 2006). Among them, volume reduction (condensation) is one of the options which enables nutrient concentration, space saving and potential inactivation of pathogens. It can be achieved by evaporation, freeze-thaw, or reverse or forward osmosis (Maurer et al., 2006; Zhang et al., 2014). During and after the urine condensation process, several factors may contribute to inactivation. If volume reduction is achieved by evaporation, both temperature (Bertrand et al., 2012) and the evaporation process itself (Ward and Ashley, 1997) can cause inactivation of microbes. Furthermore, if urine is hydrolyzed, condensation could lead to the concentration of uncharged ammonia (NH_3), a known biocidal compound (Warren, 1962; Cramer et al., 1983). And finally, urine condensation can enhance pathogen inactivation by increasing the osmotic pressure. It has been reported that the hyperosmolar conditions lead to the inactivation of pathogenic bacteria, because the raised concentration of ions, such as sodium and potassium ions, disturbs homeostasis inside the bacterial cells, and results in low enzyme activity and low penetration of metabolic products through the cell membrane (Kasumi, 2010). However, the inactivation of viruses during and after the urine condensation has not been investigated to date. For the safe use of nutrients in urine, it is important to correctly understand the inactivation profile of viruses during and after the urine condensation.

The aims of this study were therefore to evaluate the inactivation rate of MS2 coliphage, a surrogate of human enteric viruses, in concentrated urine, and to identify the relevant virucidal factors and mechanisms. First, the inactivation rate constants of MS2 coliphage in concentrated, non-hydrolyzed and hydrolyzed synthetic urine were evaluated and compared to those obtained in control buffer solutions. We then investigated the kinetics of genome loss by reverse transcription quantitative polymerase chain reaction (RT-qPCR) using six different primer sets. Finally, the inactivation data were compared to those predicted by a model of MS2 inactivation established by Decrey et al. (2015). This model was previously developed and validated for urine and diluted urine. Here, we assessed if the same model can be used to predict inactivation in concentrated urine, or if additional processes not captured by the model influence inactivation if urine components are present in more concentrated form.

2. Materials and methods

2.1. Synthetic urine samples

Synthetic urine was used instead of natural urine because of the ease to create and control the composition of concentrated solutions. Synthetic urine samples were prepared according to Griffith et al. (1976). There are three concentration levels: 1- (non-concentrated) and 5-, and 10-fold concentration. The concentrated synthetic urine was prepared by setting higher concentration of ingredients as shown in Table S1. The synthetic urine samples were poured into two 50-mL falcon tubes, and 6.5 mg urease from Jack Bean (Wako Pure Chemical Ltd.) were added to one of the bottles to hydrolyze the urea in the synthetic urine samples. Two days after preparation, the pH of the hydrolyzed synthetic urine samples was adjusted to 9.4 using 1 N HCl or NaOH solution. Similar pH conditions were obtained in all experiments using hydrolysed synthetic urine in order to minimize effects related to pH variation. The pH values around 5.2 were always obtained for non-hydrolysed synthetic urine without pH adjustment.

2.2. Buffer solutions

Ammonium and carbonate buffer solutions were prepared to serve as NH_3 and pH/osmotic pressure controls, respectively. Ammonium chloride solutions and 25% (w/w) aqueous ammonia were blended to adjust their pH to 9.4. The composition of the ammonium buffer is shown in Table S2. Sodium carbonate buffer solution was prepared by blending sodium carbonate solution (0.05 M) and sodium hydrogen carbonate solution (0.1 M) at a ratio of 1:3 (The Chem. Soc. Japan, 1966), which results in a pH of 9.4. Sodium chloride was added to the carbonate buffer solution to vary osmotic pressure. The composition of the different carbonate buffers is shown in Table S2.

2.3. Measurement of total ammonium nitrogen and osmotic pressure

Total ammonia nitrogen (TAN: $NH_3 + NH_4^+$) and osmotic pressure were measured in a subset of samples. The TAN was measured by the indophenol blue method (Scheiner, 1976). One milliliter of a sample was diluted with deionized water to adjust the ammonia concentration in the range from 0.1 mg-NH_4^+/L to 5 mg-NH_4^+/L prior to the measurement. Osmotic pressure was measured by the freezing point depression method (Brunzel, 1994). These corresponding values are reported in Table S3.

2.4. MS2 coliphage and Escherichia coli

MS2 coliphage (NBRC 102619) and *E. coli* (NBRC 13965) were obtained from the NITE Biological Research Center (NBRC, Chiba, Japan). Firstly, *E. coli* was incubated for 5 h in 10 mL of liquid broth, in a shaking water bath at 37 °C. The coliphage suspension (200 μL) was inoculated in the *E. coli* suspension and incubated overnight under the same conditions as above. The obtained suspension was centrifuged (3000 ×*g*, 10 min) to separate the coliphage from the *E. coli* cells. The supernatant was passed through a membrane filter (0.20 μm pore, DISMIC®-25CS). The coliphage suspension was stored at −80 °C until use.

The double-layer method (Adams, 1959) was used to titer the infectivity of MS2 coliphage using *E. coli* (NBRC13965) as a bacterial host. The composition of top-agar and bottom-agar is shown in Table S4. Ten milliliters of bottom agar were poured into a petri dish and solidified at room temperature. One hundred micro liters of MS2 coliphage suspension were applied to the bottom-agar and mixed with 15 mL of top-agar, which contained the host *E. coli* (10^8 colony forming units/mL). The samples were incubated at 37 °C overnight. Infective viruses were enumerated as plaque forming units (PFU) mL^{-1}, and results are reported as the average of the plaque counts of triplicate plates. The detection limit was 300 PFU/mL.