Deuterium Oxide (D_2O) on Maintaining Viability in Coliphage Bacteriophages under Low Temperatures to Model live Attenuated Viral Vaccine Additives

Summary

Viral particles are often damaged by their storage solution due to the molecular movement of the water molecules and require storage at low temperatures to reduce the velocity of the molecules. Deuterium oxide (D2O) is made with an isotope of hydrogen that increases the density of water to 1.11 g/mL by adding an extra neutron; when viral particles are stored in D2O the increased weight reduces the molecular speed of the solution, reducing trauma to the particles, increasing the temperature in which samples can be stored. A T4 bacteriophage was used to test how a viral particle would react to its environment and deteriorate over time while stored in D2O and deionized water. A sample of Coliphage bacteriophages stored in D2O was compared to a sample stored in deionized water at 16°C to determine the infectivity titer of the samples over time using a plaque assay test. The sample stored in D2O showed significantly less deterioration and slowed the rate of degradation to 6% that of normal deionized water. D2O proved to be a more advantageous solution than deionized water in supporting the health of the phages and is a promising storage additive for viral samples. This solution has application for use to increase the storage temperature of live attenuated viral vaccines, such as the Ebola vaccine, rVSV EBOV that often require storage at very low temperatures during transport and storage to remain effective and viable for administration to patients.

Research Goal and Justification

Heavy water is predicted to extend the amount of time that a coliphage bacteriophage remains viable (infectious) by slowing down the rate of degradation of the phage by reducing the molecular velocity of the water molecules that cause the damage to the viral particles.

Mass vaccination is an extremely important tactic in preventing disease outbreaks, but there are many factors that reduce accessibility to these vaccinations especially in third world countries, where they are needed most. This research addresses one possible way to increase the accessibility of these medications by exploring a new vaccine storage chemical that could increase the lifespan of the vaccine and allow it to be stored at high temperatures to avoid the difficult task of transporting it at -40 C. If transportation for this vaccine is made easier, more people will have access to it. This research could also be applied to bacteriophage therapy and future virus based medications.

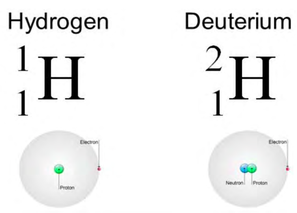
Introduction

Live vaccines work by taking a sample of a virus and engineering it so the body recognizes it as an invader and begins to make antibodies against it, but it does not infect the body and make it sick. The base of the vaccine is still a live virus and it needs to be kept in specific conditions to stay viable and infectious. Some vaccines are very unstable and have to be kept at very low temperatures to stay viable for long periods of time. Vaccines like this are very difficult to transport and lead to an issue in what is called the Cold-Chain, keeping a medication at perfect conditions from manufacturer to patient.. The solution to this has been different additives into the solution which a vaccine is transported in, in attempt to make it more stable. Bacteriophages are small viral particles that infect bacteria and cause adverse effects, just like a virus infects a human.

Bacteriophages are also a good model for how viruses survive and react to their environment. They are an adequate model to test how another virus would response to additives and or changes to the solution it is stored in. Normally, when vaccines are stored in water, the molecular movement of the water particles damage the virus and reduce its infectivity, Making the vaccine less viable, and effective.

D2O slows down this damaging movement while also providing a suitable environment for the virus. D2O has been proven to extend the life of vaccines when exposed to heat, but has yet to be tested as an additive to stabilize in colder conditions as well. Since D2O is simply made of an isotope of hydrogen, it is completely safe in the body and behaves virtually the same as water which makes it biocompatible in humans and will cause no adverse reaction.

A cold box for the transport and storage of vaccines. *Bulletin of the World Health Organization*, *56*(3), 427–432.



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Applications

Vaccine thermostability is the leading impediment that stops vaccines and other medications from being transported to areas of the world most in need. D2O has the potential to increase the thermostability of these critical vaccines and other viral based medications such as phage therapies and viral treatments. If these medications were to be made more stable, they could potentially save thousands of lives because it would be available to more people, cost less money, and be easier to move.

If vaccines alone be stored and transported more easily, millions of dollars could be saved from reducing vaccine wastage, transportation costs, and equipment usage.

(Meera Senthilingam,"Experimental drugs approved for use in Congo Ebola outbreak" CNN)

But more important than saving copious amounts of money, increasing the thermostability of vaccines will lead to the saving of millions of lives because the people affected aren't just dying from common illnesses, they are dying from completely preventable illnesses. Illness that could be eradicated from entire countries if the necessary vaccines could get there.

Although the most beneficial use of D2O at this point in time is vaccine stability, it can also be used to store bacteriophages and viruses for lab testing, reducing the cost of research for future medications.

Bacteriophage therapy or phage therapy as it is more commonly called, has the potential for becoming the next generation of antibiotics to combat the antibiotic crisis that resulted from the overuse and overprescription of these valuable medications. In the future, the world and especially the US will have built up such resistant bacteria, that antibiotics will become obsolete and new medications will be needed to treat bacterial infections.

All graphs and images were student developed unless otherwise cited.

Original Infectivity Titer Determination Via Plaque Assay

The bacteriophages were stored in one solution of mainly D2O and one solution of deionized water. The viability of the phage sample was determined by performing a plaque assay test which shows the original infectivity titer of the original phage sample.

The original phage sample was stored in a solution of 3 mL tryptone broth at an infectivity titer of 10^4 particles per mL and was refrigerated until dilution so the sample did not degrade by an appreciable amount. The sample was diluted to 100 phages through serial dilution with deionized water where 1 mL of the original phage sample was placed in 9 mL of deionized water and then 1 mL of this solution was placed in another 9 mL of deionized water. This process was completed one more time until the phage dilution was 101 particles per mL. 1 mL of this final solution was then placed in one sample of 9 mL deionized water to make the deionized sample and 1 mL was placed into 9 mL of heavy water to create the D2O sample. Both of these samples were stored in sterilized test tubes and immediately placed into the medical freezer at 16° C at time 0.

A plaque assay was performed to determine the infectivity titer over time for each of the samples. The *tryptone Base-layer agar was melted in a water bath at 97 Celsius and then poured into four sterile petri dishes until a 5mm thick layer was coating the bottom. Petri dishes were left to harden and cool for 2 hours on a clean surface away from drafts. A tryptone *Escherichia coli* host solution was made by warming a 5 mL sample of *tryptone broth to 37 Celsius and placing it onto a *tryptone slant agar host of *Escherichia coli* and teetering the solution back and forth until the broth is cloudy with cells. The host sample was then placed into a sterile test tube, sealed, and refrigerated until needed.

Two sterile tubes of 4 mL* tryptone broth were brought to room temperature and 0.5 mL of tryptone *Escherichia coli* host solution was added to each of the tubes and sealed. Both tubes were incubated at 37° C for 30 minutes, swirling each tube every 5 minutes to enhance growth. After 30 minutes elapsed the samples were inoculated with 0.5 mL of each of the phage dilutions (one in deionized water and one in D2O) to bring each tube up to 5 mL total. These test tubes were incubated for 20 minutes at 37° C, swirling every 5 minutes to enhance growth.

While the samples were incubating, two tubes of 2 mL *tryptone soft agar were melted at 97° C and then cooled to 45° C for mixing with sample. After the sample finished its second incubation, 1 mL of each phage and bacteria solution and 0.3 mL of *E. coli* host solution was mixed with the soft agar then immediately poured onto each petri dish and teetered to cover the plate. The dishes were quickly covered and left to sit and harden for 15 minutes before being placed lid down in the incubator for 16 hours.

Once the petri dishes had been incubated for 16 hours, they were removed and refrigerated for further analysis. Assays were analysed under 40x magnification by counting number of plaques in the field of view of a representative sample of the whole assay and then mathematically estimating the amount of plaques on the entire assay plate. Once the number of plaques had been estimated for the entire plate, the number was divided by the original dilution (100) to get the infectivity titer of the sample when it was assayed. This is the number that was analysed to determine degradation in each sample. Each dish was also photographed under 40x magnification to see qualitative degradation in size and shape of plaques formed to observe overall health of phage sample. The infectivity titer and the magnified images of the phages are used in conjunction to determine how effective each solution was at protecting the bacteriophage from damage.

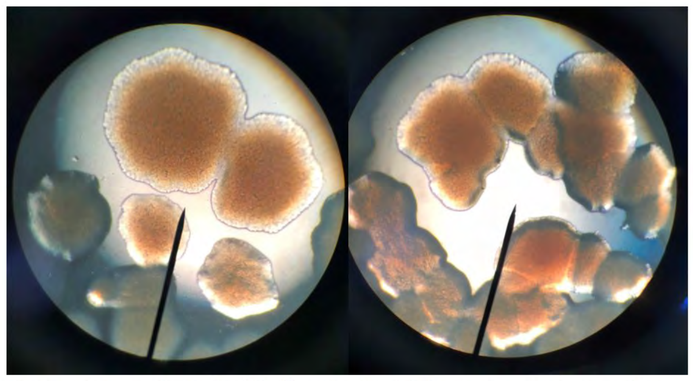


Figure 1: Day 1. Left: D_2O sample Right: Deionized sample.

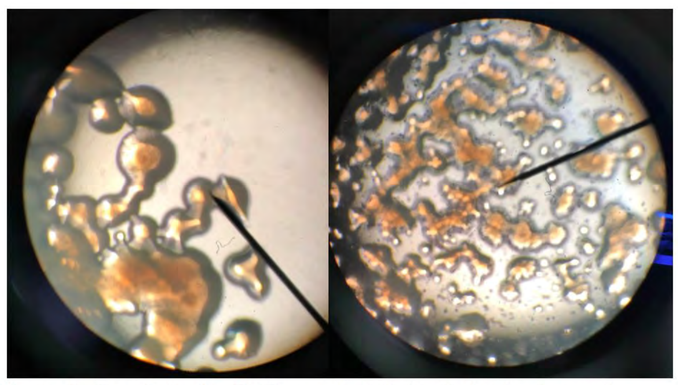


Figure 2: Day 3. Left: D_2O sample Right: Deionized sample.

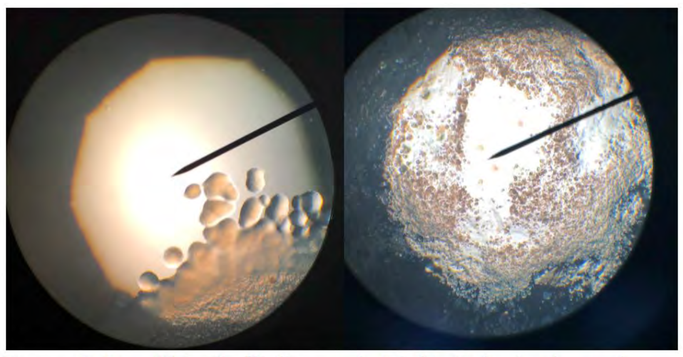


Figure 3: Day 10 Left: D_2O sample Right: Deionized sample.

Degradation Regression Results and Analysis

Over the testing period of 10 days, both samples showed signs of degradation both qualitatively and quantitatively. The sample stored in deionized water degraded at a higher rate than the sample stored in heavy water (Figures 4&5). At day 1 both samples produced large plaques that were bulbous and mostly round with smooth borders (Figure 1), but by day 3 the samples already showed signs of deterioration (Figure 2). The deionized sample produces much smaller plaques and with jagged borders and irregular shapes. The D2O sample produced smaller plaques with slightly irregular borders but showed significantly less deterioration than the deionized sample. By day 10 (Figure 3) the deionized sample was nearly entirely deteriorated; the plaques were unrecognizable and therefore the sample's infectivity could not be counted and was zero by default. The D2O sample produced even smaller plaques but maintained their shape and even borders.

Since the degradation in infectivity titer and qualitative health of the phage consistently decreased over time, one can see how storing the phage sample in D2O is preferable to deionized water.

Graphical analysis was performed to show the deterioration of the phages over time, and the change in infectivity titer over time. Error bars allow for up to 20% error in infectivity titer to account for the possibility of phage miscount in each assay. Since no error bars overlap on the graphs, the difference in degradation is statistically significant and has an extremely low chance <5% probability of occuring by chance.

By analysing the slope of deterioration in the regression graph of infectivity titer (Figure 6), it was determined that the D2O sample degraded at approximately 100 phages per day and the deionized water sample degradation 1700 phages per day. Each regression and correlation coefficients of .95 and .99 respectively so the data has a high correlation and the graph is a valid way to determine degradation rates. The D2O sample degraded at 6 +/-1.2 % the rate that the deionized sample deteriorated. This is obviously a very significant difference in degradation, showing that D2O was a more preferable storage solution than deionized water based on the infectivity titer over the testing time period.

The qualitative health and infection effectiveness of the bacteriophage also went down over the testing period. At day one (Figure 1) the phages in both samples displayed large rounded plaques on the agar, implying that each phage was very effective in infecting the bacterium and lysed the surrounding cells evenly. This shows that both sample started at the same qualitative health level and weren't damaged to begin with. The D2O sample started at an infectivity of 4.0 x 104 PFU and the deionized sample started at an infectivity of 1.6 x 104 PFU. Even though each sample started at a different titer, degradation is measured relative to the starting titer, so the difference is negligible.

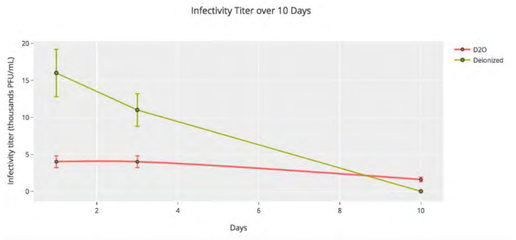


Figure 4: Graph of Infectivity titer over time (10 days)

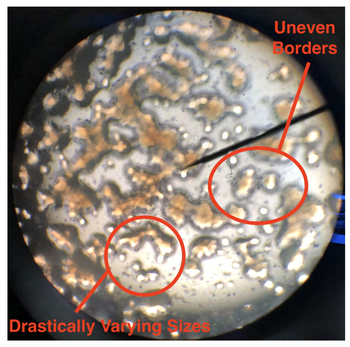
Figure 5: Graph of change in infectivity titer over time (10 days)

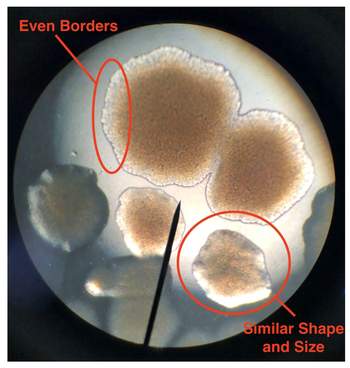
Figure 6: Regression graph of change in infectivity titer over time (10 days)

Degradation Regression Results and Analysis (Cont.)

By day 3 (Figure 2) the D2O sample plaques were smaller and had more variance in their edge shape but remained fairly rounded with even barriers. This shows that the phages in this sample were slightly less healthy (even though the sample showed no appreciable infectivity titer degradation) because when the phage went through the lytic cycle in the agar it did not expand as equally in all directions. By creating plaques of more irregular shapes, the day 3 results for D2O show a qualitative reduction in health of the phage, but this reduction is very small compared to the degradation observed in the deionized water sample. This sample formed very small plaques with heavily irregular borders. while this sample also showed degradation in it's infectivity titer (1.1x 104 PFU), it had severe physical degradation as well. The irregular borders show the declining health/infectivity of the phage because the phage is not producing the even circular lysis bloom of progeny that one would expect from a healthy phage. Instead, it is lysing in heavily irregular patterns indicating poor health and inability to properly infect the bacterium.

At day 10 (Figure 3) the D2O sample produced smaller plaques than it's day 3 test and had a decline in quantitative infectivity titer (3.1 x103). The plaque's decreasing size was indicative of declining phage health, but despite the size difference, the plaques still maintained their mostly circular shape meaning that they were still infectious and viable. The deionized water sample was completely degraded by day 10. There were no discernable plaques and the sample formed random spots of infection with very irregular (if any) borders. Although the phages were still able to infect extremely small amount of bacterium they were too weak to form a recognizable plaque. This sample was determined to be entirely degraded despite the minor spots of infection because although there were a few phages that were still viable the sample was too unhealthy to be deemed viable in its entirety. If this sample had been an actual vaccine, it would not be potent enough to produce the desired effect of immunity and would be insufficient in alerting the patient's immune system. Since this is a model experiment of how a real vaccine would behave under these circumstances, this sample was deemed unviable and completely degraded because of this reason.





After the graphical analysis was performed on these results, it was found that the degradation difference was statistically significant and the addition ofD2O to a vaccine storage solution greatly impacted the rate of degradation of the sample both in infectivity titer and qualitative health of the phage. The sample stored in D2O did not only degrade more slowly than the deionized water sample, it preserved the overall health of the individual phages better than the deionized water when analyzed under magnification.

Conclusion

D2O is a viable additive for vaccine storage solutions, and could aid in the reduction of problems that originate in the Cold-Chain. Since the main challenge facing mass vaccination in third world countries is availability of vaccines, making transportation of these critical medications easier is a vital step in preventing epidemics.

Vaccines still faces challenges in the Cold-Chain due to the extremely low temperatures that most of them need to be kept at. By increasing the length of time that the vaccine can remain viable and the temperature it can be stored at, the vaccine can be made available to larger groups of people in need.

D2O proved to be a possible solution to the barriers hindering mass vaccination in third world countries by prolonging vaccine potency for a much longer time period than simple deionized water. This extension of storage time could allow for more remote areas to be reached and less vaccines being wasted due to improper transportation, storage, or accessibility to patients.

This experiment modeled how vaccine potency could be extended by storing it in D2O compared to the deionized water that it is normally kept in. The degradation of the infectivity titer of each of the samples supported D2O as a much more suitable storage solution to deionized water because the reduction in infectious phages was significantly lower than the standard deionized water sample. D2O is also an ideal storage solution because it has nearly the same molecular interactions as water so it can be combined with other additives to further increase stability. D2O also supported the qualitative health and infection effectiveness of the phages as seen through magnification analysis.

Future Research

This additive needs to be first tested with an actual sample of the rVSV EBOV vaccine to ensure that this experiment is an accurate model for how this vaccine would respond to being stored in heavy water. Then it has to be tested under many different conditions that would be present during the transport of the vaccine.

Vaccines are exposed to temperature fluctuations during transport and D2O needs to be evaluated at different temperatures to determine first if it is as effective at other temperatures, and second if the solution can maintain its stabilizing effect while also tolerating temperature changes. If it can tolerate both of these factors then an ideal temperature zone needs to be determined where D2O maintains the most infectious potency while also being stored at a temperature that enables easy transportation of vaccines.

This additive also needs to be tested in conjunction with other vaccine storage additives to test for any possible interactions between the two. Although it is very unlikely that D2O would interfere with any molecular processes in the other vaccine stabilization additives, this still needs to be verified.

In addition to testing for interactions between other additives and D2O, the safety of the additive needs to be assessed in humans. As with other additive interactions, it is extremely unlikely that D2O could cause an adverse reaction in people due to its inert nature, and having nearly the same chemical makeup as water, but it still needs to be verified. The safety of heavy water in humans has already been determined as completely safe through oral consumption, but the safety must be confirmed via the intravenous route.

Economic research must be conducted as well to determine the cost effectiveness of replacing deionized water with D2O in individual vaccine doses. Although D2O does add an additional cost to each vaccine dose, it would relieve significant financial stress from the transportation process and it would allow for less vaccines to be wasted. Despite these advantages the financial benefits of using D2O in place of deionized water needs to be evaluated.