

Use of Enteroids to Evaluate Persistence of Infectious Human Norovirus in Seawater

[Announcer] This program is presented by the Centers for Disease Control and Prevention.

[Sarah Gregory] Hello, I'm Sarah Gregory, and today I'm talking with Dr. Marion Desdouits, an environmental virology researcher in France. We'll be discussing the persistence of human norovirus in natural seawater.

Welcome, Dr. Desdouits.

[Marion Desdouits] Hi, thank you.

[Sarah Gregory] Let's start off with what is norovirus?

[Marion Desdouits] So norovirus is a small virus that mainly causes acute gastroenteritis in people. It's called often the stomach flu or the winter vomiting disease, and actually many people have encountered this virus during their life. It's been discovered following an epidemic in a school in Norwalk, Ohio in 1968. So that's where it got its name from. Norwalk's a norovirus and it's a genus of several viruses among the family of *Caliciviridae*. And this family is characterized by a short RNA genome, which is roughly one-fourth of the SARS-CoV-2 genome. So it is a really small virus. And also the viral particles are very small because they measure something like 30 nanometer.

So the virus infects people, otherwise we wouldn't be talking about it, mainly. So human norovirus, that infects people. But there are also noroviruses that infect other mammals like pigs, cattle, cats, dogs, and marine animals. In the past, it was—and still nowadays—it started by the detection of its genetic material with like the PCR tests similar to the one that's done on SARS-CoV-2 nowadays (and many people have heard of). There are animal models that exist using mainly the murine virus that infects mice, because this virus is able to replicate in vitro in cells in culture, so it's very easy to use. But for human norovirus, for a long time there was no in vitro model, so we could not replicate the virus in vitro. So there was a lot of questions that we could not address. And there were some studies that were done on human volunteers because that was the only way to assess whether the virus was infectious or not.

[Sarah Gregory] And what are human intestinal enteroids?

[Marion Desdouits] So these are actually adult intestinal stem cells. So they are derived from intestinal crypts that are taken from the tissues of ASC donors. There are often people that undergo bariatric surgery and then their healthy tissues are used to make the human intestinal enteroids that can grow in vitro in 3D images. So we have them in a culture in cubicles, and they grow in a very rich medium full of niche factors and growth factors that allow them to remain stem cells. And then we can also differentiate them so they become like very little cells so that we can replicate a gut. It's a very physiological model of the gut. That's why I said up in the later 20s, it's used nowadays to understand a lot of the gut functions. And in 2016, the team of Mary Estes in the Baylor College of Medicine in Houston, Texas has published very important data that showed that human norovirus can replicate in these cells (human stem cells). And now this allows us to study if the norovirus can stay infectious by looking at whether it can infect the cells or not in vitro.

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[Sarah Gregory] And you mentioned earlier some different types of noroviruses. Can you quickly tell us about the different kinds?

[Marion Desdouts] Yes. So indeed, norovirus is highly genetically diverse. So the genus has a lot of different genogroups inside, and a different genogroup can infect either people....so, genogroup I and genogroup II, for example, can infect people but other genogroups can infect other mammals. And inside the genogroups, there are also many, many genotypes (like more than 50). And there are also tentative genogroups and genotypes because there is still a lot of diversity to be discovered in this genus of viruses.

[Sarah Gregory] And do these different types affect people differently?

[Marion Desdouts] Yes. So it's a very interesting question. Actually, norovirus detection and genotyping characterization of the genome are not different systematically in sick people because gastroenteritis can stop quite fast, so people don't always undergo a lot of tests. But even though in the past decade we observed that one genotype was more often found in people...so it's the GII.4 genotype that's very, very more frequent than all the others. So there might be several reasons why other genotypes are less frequently detected. It could be underestimated because they are less pathogenic so the people do not seek healthcare, so they are not tested so then we cannot see these viruses. Or maybe also they can affect some specific populations (like children), or probably also they are just less well transmitted in the human population, so there are less variance.

[Sarah Gregory] And how is norovirus spread?

[Marion Desdouts] So norovirus is mainly spread from person to person. This is described as the fecal-oral route, because the virus is secreted in the stool of infected people, sometimes for several weeks. It can be rather long. And so, good hand hygiene is very important there. So we believe that the virus can be transmitted from touching surfaces or from direct contact between a sick person and the receiving person. But there is also very important new research that has gone out two weeks ago that suggests that the transmission can occur through saliva. There is current evidence in mice, and it has seemed to be a concern in humans. But this could explain some things that were quite weird about the way norovirus can spread in humans. So fecal-oral route, hand hygiene, but maybe also saliva is possible.

And there are also other modes of contamination that is not directly from person to person, but through contaminated food and water, like ready-to-eat food that can be contaminated by food handlers that are sick and that have bad hand hygiene. But also, there are fresh produces like shellfish, berries, or salad that can be contaminated during the production when the waters that are used are contaminated. So what's very interesting here is that there are differences in the transmission mechanism between the different genotypes. As I said, the GII.4 genotype is the most frequent in the human population. And it's also more detected in large outbreaks with human-to-human transmission, like in both cruise ships and healthcare facilities also, where the density of people is high. And there we can find a lot of GII.4 genotype. But other genotypes, for example, like the GII.3, GII.6, G.I.I, are more detected in waterborne or shellfish-borne outbreaks. So there seems to be a kind of selection in the mode of transmission.

[Sarah Gregory] And you mentioned particles earlier. Are norovirus particles particularly infectious?

[Marion Desdouts] Yes, actually they are. So the first studies estimated the infectious dose would be around 10 viral particles, so that's very, very low. So there are more recent estimations that convert around 100 to 1,000 particles to establish an infection in humans. But this is still quite low compared to other viruses. What makes them also particularly infectious is that the virus is known to be very resistant. So it can stay in the environment or on surfaces and is believed to remain infectious for a very long time. So that also increases the risk to be contaminated.

[Sarah Gregory] Your article talks specifically about shellfish being a main source of contamination. How does this happen?

[Marion Desdouts] So shellfish are one of the main foods implicated in norovirus foodborne outbreaks in Europe and also in the US. So it's not a main source of contamination because it's rather low compared to person-to-person. But among the foodborne epidemics, it's really a striking phenomenon that shellfish are often implicated.

So how does it happen? The cycle is quite simple. So the virus is excreted in stool by infected people, then it goes to sewage and the sewage is partially treated, or they can also....the sewage can spillover. And there it can reach environmental waters like your face water or coastal waters when you are near the seashore. And there, you can have contamination of the shellfish during the production because the shellfish filter feed. So they filter large quantities of water from which they take out the food, which are little particles of algae, for example. But doing that, they also capture the viruses that are in the water. Another reason is that most shellfish are eaten either raw or very lightly cooked, and oysters in particular are eaten raw. So there is no cooking and the infectious risk is very high. Another reason, finally, especially for oysters, is that oysters express a molecule in their tissues that is very similar to the one that is known to bind norovirus in humans. So in humans, norovirus is known to interact with a little molecule which is of the sugar family that is called the histo-blood group antigens. And these molecules, there is one that is very similar in shellfish. So this should be believed to contribute to the accumulation of the virus in the shellfish tissues. So shellfish are not infected because the virus is specialized to humans, so it cannot infect shellfish. But the shellfish can accumulate the virus, and 10 times more concentrated in the shellfish tissue than in the water surrounding it.

[Sarah Gregory] You mentioned some ways that this norovirus contaminates seawater. You want to go over that a little bit more again?

[Marion Desdouts] Actually, norovirus is not so concentrated in the seawater itself, so there is no or very weak risk when bathing. And it's there only in areas that are submitted to sewage discharge, so it's not all the seawater. And especially not in areas where people go for recreational activities and sunbathing and stuff like that. And another characteristic of the contamination is that it's usually very transient, like the sewage spillover comes by and then goes away. But the shellfish that are there, then we can concentrate and integrate the contamination and keep it for a very long time. So it's the concentration in quantity but also in time. So that's why the risk is really increased in shellfish in particular rather than in seawater.

[Sarah Gregory] I see. So it's dangerous for people that eat the shellfish. The shellfish don't get infected themselves, and swimming in those areas is actually okay.

[Marion Desdouts] And in US and in Europe, there are a lot of surveillance of shellfish-producing areas. So shellfish that are sold for consumption are usually contamination-free. So

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there can be accidents, but there is a lot of work that has been done to check on the existence of contamination, and especially norovirus.

[Sarah Gregory] How was persistence of these infectious human norovirus in shellfish investigated previously?

[Marion Desdouts] So the first data that were used to estimate how long the virus can stay infectious in shellfish were actually epidemiological data and case studies where we could use the delay between the contamination events and the consumption. For example, when shellfish were known to be kept in a separated tank on the shelf for several weeks sometimes, we know that the contamination occurred before. And then when they are eaten and there are sick people after that and confirm that norovirus is really implicated and was present in the shellfish, we can say, "Oh, so the virus stayed infectious for these several weeks in the shellfish tissue". So case studies like that show that the virus could stay infectious for quite a long time.

After that, the field has turned to more mechanistic studies. And since norovirus was still not culturable easily, we've been using a related virus (that's the Tulane virus). So the Tulane virus belongs to the same family (the *Caliciviridae* family) than norovirus, but to another genus (the *Recovirus* genus). And so it's a similar virus; it is easily culturable; it has also a fecal-oral transmission mode; and very importantly, it binds similar ligands in norovirus. So it's a very interesting model because then we can have the ability to take into account how the virus interacts with the shellfish tissue. In our lab, we have detected that this virus really behaves in oysters like norovirus. And so previous studies have showed that this Tulane virus can subsist for up to three weeks in shellfish tissue using a quite high initial dose. But it did show that the virus can stay infectious for several weeks.

[Sarah Gregory] Why did you want to do this study?

[Marion Desdouts] These studies with the Tulane virus have some limitations. First, it's believed that norovirus may be more stable than the surrogate viruses that are used to replace it when we cannot do direct experiments on norovirus. Then people have been using Tulane virus or murine norovirus, and we know that it's possible that norovirus may be even more stable than these viruses. So then you underestimate the counter measures that you should apply. Also, Tulane virus doesn't have the genetic diversity that is estimated in norovirus. And then also, of course you cannot compare different norovirus genotypes if you use Tulane virus. So we could not ask this to the question of whether different genotypes were selected in the shellfish and in the environment because they are not as stable (all of them).

And then, also the enteric model (the human intestinal enteric) was finally accessible to allow direct assessment of infectious human norovirus. So for all these reasons, we wanted to undergo this study to check how long norovirus could remain infectious in the environment. So we could not directly go to shellfish because the protocol to recover the virus from shellfish tissue is not ready yet for infectious virus, which is quite challenging. But we had a protocol ready to recover it from seawater, so we started with seawater. It's the last step before shellfish contamination. It's an interesting proxy to estimate how long the virus can remain in shellfish, because it would be the same temperature, the same salinity. So that's why we first focused on seawater. And we used norovirus and Tulane virus to check how long they can remain infectious.

[Sarah Gregory] You used enteroids to evaluate human norovirus in the seawater. How did you do that?

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[Marion Desdouts] So enteroids, as I said, they are cultured in vitro in a gel. And they were given to us by Mary Estes, so the inventor of the norovirus culture model. She also shared with us all the regions and protocols. So thanks to her, we were able to establish the enteroid culture in our lab, which was quite challenging. I must say that when you infect a cell with a virus, often you would think that the cell is going to die, so you can just look at your culture and say, "Okay, the virus is there, and the cells died". It's very easy. That's the case, for example, for the Tulane virus. But norovirus and enteroids, by eye you don't see anything. On the microscope, there is no effect on the culture. So this is a bit tricky because it means then you need to measure the amount of virus in your culture to know whether it's really replicated or not.

So how do we do that? We have a duplicate infection. We put the virus on two cultures at the same time, and one of them is stopped after one hour. And this is the baseline attached virus on the cell that we use as a reference. And then the second culture is kept for three days to allow the virus to grow in the cell. After the three days, we also stop the culture by freezing it. Then we extract the viral genome from all the cultures. We quantified the amount of viral genome using the PCR. And we compare the condition after one hour and the condition after three days. And if the virus did replicate in the enteroid culture, we should see more genome (viral genome) after three days than at one hour. And this is how we can say that the virus is really replicating and that it is still infectious.

[Sarah Gregory] Is there anything else you want to tell us about how you conducted this study?

[Marion Desdouts] Yes. So I told you about the enteroid issue and how we used them. But actually, before using the enteroids, what we did is to have fresh seawater from the French shore that were sand filtered, and then we put virus in the seawater. And so, we used three different viruses: so the Tulane virus, because we wanted to have the comparison with the previous work using this virus, and then we used two human norovirus genotypes: so, genotype GII.4, which is the main virus circulating in the human population, as I said already, and the GII.3, which is rather frequent but less than GII.4, and is interesting because it's often found in foodborne epidemics. So we wanted to see whether this virus (GII.3) was more detected in shellfish or berries or salad maybe because it was more stable than GII.4. That was our main hypothesis.

So these different viruses, the seawater was split in little tubes that we kept at 12 degrees in the dark. And then, we did random sampling of these tubes for several weeks. And we quantified the genome of the viruses in seawater, and we also put inside the infectious virus and inoculated them with enteroids. And then, as I said, we quantified using the PCR and we compared one hour versus three days of the culture. And we did three different experiments with three different water samples.

[Sarah Gregory] And after all this, what did you find? Did you find out how long noroviruses last in seawater and in these shellfish?

[Marion Desdouts] Yes. So we had a very interesting result, we think. The two first experiments showed that norovirus genome was very stable, and the Tulane virus at the genome level was already less stable than norovirus. And actually, if we looked at infectious virus (not just the genome, but the full virus that is replicating or not), we could detect infectious norovirus for up to five weeks in seawater and Tulane virus for up to three weeks, like we did in shellfish. So here we saw that norovirus was more stable than Tulane virus.

But the last experiment was a bit different. So there was a fast drop in the genome level for both viruses, and an even faster loss of infectious virus. Norovirus (both noroviruses) were still more stable than Tulane virus, but they were really, really less stable than in the first experiments.

[Sarah Gregory] Was any of this a surprise to you?

[Marion Desdouts] Yes, it was very surprising on several levels. So the first surprise was that we had similar results between the GII.4 and the GII.3 human noroviruses. As I said, we supposed that GII.4 was maybe less resistant, and that's why we see it less in foodborne or waterborne contamination. But this was not the case here, because both viruses had really the same behavior. So we think it's possible that enteroids may be more sensitive to GII.4, so then we overestimate the ability to persist (or more accurately, we underestimate the ability of GII.3 to persist). Or maybe also because we only tested one condition (seawater at 12 degrees) and maybe a different temperature or salinity or maybe in shellfish, this stability would be different between the two viruses. So there's more research to be done, as always.

The lesser stability of Tulane virus was never shown directly, but it was suspected. So that was not really a surprise. But the very big surprise was how variable the result would be between seawater samples that were of the same origin that were sand filtered, and rarely we see a very different result in the same condition (12 degrees in the dark). So this opens up a lot of new questions as what in the seawater drives and impacts so much on norovirus stability and persistence.

[Sarah Gregory] As with any new investigation, I imagine there were challenges. I think you mentioned one before. Can you tell us what some of them were?

[Marion Desdouts] So as I said, establishing the enteroid culture in the lab was quite challenging. Although we had a lot of help, with the Atlantic Ocean separating us, it was not very easy always to have the good ways to enteroid cells, etc. So it took quite almost a year to have the system running. But thanks to the help we got, we were very happy to finally have good results. Experimenting stem cells is also a high burden of work, because we had to have enteroid cells ready to be infected at each time point. So we had to come on weekends to prepare the cells, we had to do a lot of cell culture and then a lot of PCR to measure viral genomes. So for a small team like ours, it was a lot of work, but it was really rewarding. And the last challenge was the variability issues in the seawater. So after the three experiments and seeing how the third experiment was so different from the two first, we really tried to understand what happened and to overcome this variability issue. But then finally we understood that it was very important information actually, because it really shows that if you use natural seawater, there are lots of different things inside that could impact norovirus stability. And this is very important research to be conducted to really understand how the nature of the seawater could have an impact on the quality of the shellfish where they are grown.

[Sarah Gregory] Would you consider that the most important public health implication of your study?

[Marion Desdouts] Yes. It's a very important implication of the study because knowing how the virus can persist in the environment, then we can design adequate countermeasures to handle the contamination. So we showed that norovirus are highly stable in seawater. And another important result is that the surrogate virus (Tulane virus) was actually underestimated in this study. I think it's also important to keep in mind when designing these countermeasures, because

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then if you use the data on surrogate viruses, you risk underestimating the stability and not being strong enough to counter norovirus (for example, the current countermeasures that are applied on shellfish).

So it could be either the deprivation—that means that when the shellfish have a high burden in bacteria (in fecal bacteria) which can happen if the seawater was tainted by sewage—then the shellfish are taken out of the seawater, and they are put in tanks on the shore and in clean seawater that is purified and cleaned. And then for a few days it's enough for the bacteria load to go down. But if we know that it's probably efficient on norovirus, at least at the genomic load and in the presence of viral genome, it is not affected by this countermeasure. So understanding how long the virus can subsist in this condition, maybe we could increase the duration of the deprivation process, or we could just know that it's not going to have any effect and now we have the proof for that.

And also, the second important countermeasure (at least in Europe) is that the pollution areas for shellfish can be closed for several weeks when there is a proof of norovirus contamination. And this number of weeks actually needs to be based on the actual risk. So it needs not to be too long, not to impact the producers too much. But it does need to be long enough for the norovirus to be deactivated. And what our study shows is that several weeks is really needed, actually.

[Sarah Gregory] How do you hope this information from your study will be used going forward? More studies or, like you said, more guidelines?

[Marion Desdouts] I think before really having guidelines we need more studies. This is just a first study using this system, so it gives a lot of information but it's still on a limited number of experiments, a limited number of conditions also, because we only tested one temperature, for instance. So I hope that with this study, there will be other teams that are going to use this kind of enteroid to study norovirus persistence, maybe in other matrixes or in other conditions to compare with our conditions. And we need more information on the norovirus persistence to calculate the half-life to estimate the impact of the temperature, the impact of the virus strain, especially be able to consolidate the regulations on the crucial production areas and guidelines for producers.

[Sarah Gregory] Let's just talk about norovirus as an illness for a little bit here. What are the signs and symptoms of it?

[Marion Desdouts] Well, it's very classical acute gastroenteritis—vomiting, diarrhea, fever, abdominal cramps. Really, the classical experience by many people. And actually, there are millions of cases worldwide of norovirus, because it's the main cause of acute viral gastroenteritis. And it's estimated one million hospitalization worldwide per year and an economic burden of 60 billion dollars worldwide. So this is really...actually a common pathogen and a common disease.

[Sarah Gregory] Does anyone die of it?

[Marion Desdouts] Well, most of the cases are self-resolving in a few days. But sadly, there are more complicated cases. So for example, there can be chronic infection of immunocompromised people. So they don't die often, but it can really impact on their quality of life. But the virus can also be life-threatening for the elderly, and especially in children in developing countries

(through dehydration, mostly). So the virus, it causes (we estimate) 200,000 deaths per year worldwide, mostly in developing countries.

[Sarah Gregory] And once somebody has norovirus, is there a treatment?

[Marion Desdouts] There are no specific treatments, so people can always use painkillers or antipyretics to let the fever go down. But the main treatment is rehydration, because that's the main risk (to be dehydrated). And a vaccine is under development, but it's not available yet.

[Sarah Gregory] A vaccine would be interesting since there's so many different types of it, right?

[Marion Desdouts] It's very difficult to set up for this reason. I think they are mostly targeting the GII.4 genotype.

[Sarah Gregory] Are there ways people can protect themselves from getting it?

[Marion Desdouts] So since the contamination goes from hand to mouth, good hand hygiene is very important. Especially if you are symptomatic, you need to be very careful. But there are also asymptomatic infections. Also, the virus excretion can last for several weeks, even after the symptoms have been resolved. So actually we need to be always careful and to keep good hand hygiene all the time. What we notice also is that the countermeasures that were enforced during the COVID emergence were able to dramatically drop the number of cases of gastroenteritis and also with norovirus circulation. So social distancing and wearing masks also help against norovirus.

[Sarah Gregory] Yes. Wearing a mask, you wouldn't be touching your mouth, then, with your dirty hands, right?

[Marion Desdouts] Yes, exactly. You don't touch your mouth with your hands so much. And with this new research about norovirus in saliva, it also sheds light on another possible mechanism.

[Sarah Gregory] I learned recently that *C. diff* isn't affected by hand sanitizer, that you actually have to wash your hands with soap and water. Do you know if norovirus can be killed with hand sanitizer?

[Marion Desdouts] So enteroids are used to check here on this kind of question whether norovirus were efficiently deactivated by alcohol. And actually chlorine is much more effective, and alcohols are not very effective to decontaminate norovirus. So there were no specific hand sanitizer formula that was tested on norovirus that I know of. But since the virus resists quite well to alcohol, it probably is not very efficient.

[Sarah Gregory] So places that have hand sanitizer stations outside of food areas, that's not really going to help with norovirus, then?

[Marion Desdouts] Well, it's always better than nothing. But it's probably not the best one. And it's still efficient against bacteria, so it's still good practice.

[Sarah Gregory] Well, tell us about your job and your career path and where you work.

[Marion Desdouts] I am an environmental virology researcher. I joined the Health, Environment and Microbiology Lab in 2016. So this lab is part of the Ifremer French Institut for Marine Research. So that's why we are looking for the coastal environment, and in our lab we are specialized in studying the human pathogens in the coastal environment. So myself, I have a PhD

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in biology that I got at the Pasteur Institut in Paris in 2011. And for this, I was studying the interaction between muscle cells and human viruses HTLV-1 and influenza-1 (so, influenza A) —so older viruses. Then I went to the Pasteur Institute of Bangui in Central African Republic for a short stay, where I studied the evolution of yet another virus (the chikungunya virus) in Central Africa. And then I joined as a postdoc the Curie Institut in Paris from 2012 to 2016, where I worked on HIV interactions with macrophages, and yet another virus but a very famous one.

And so nowadays I focus on enteric viruses, and I mainly work on norovirus. And the main question that drives my research is how is the virus transmitted through the coastal environment; what is specific of this mode of transmission through shellfish; if the virus is selected, is it a source of new diversity; and then, through which mechanism does all this happen?

[Sarah Gregory] Well, you have a very varied career. What's your favorite thing you have ever worked on?

[Marion Desdouits] That's a tough question, because actually I had fun in all of them. So I think all viruses are very fascinating. I really enjoyed my postdoc on HIV because HIV is a very well-known virus, so the community is very wide, and you learn new things and very important things all the time. It's a very fascinating field. But my current position in norovirus is really interesting, too. These are very interesting viruses because of this type of diversity and their mode of completion also, which are varied, also very fascinating. So I am not sure I have a favorite. Maybe I am just happy doing what I do. I guess I'm very lucky.

[Sarah Gregory] You are very lucky, and you do have a very interesting career.

Well, thank you so much for taking the time to talk with me today, Dr. Desdouits.

[Marion Desdouits] Thank you very much for having me and giving me this opportunity to widen the question we are working on.

[Sarah Gregory] And thanks for joining me out there. You can read the July 2022 article, Use of Human Intestinal Enteroids to Evaluate Persistence of Infectious Human Norovirus in Seawater, online at [cdc.gov/eid](https://www.cdc.gov/eid).

I'm Sarah Gregory for *Emerging Infectious Diseases*.

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