Cryptosporidium parvum: Infectivity in Healthy Volunteers and Laboratory Animal Models
The first thing I would like to do is to share with you the number of people that it takes to do this work, and this is really a short list, but these people are especially important. The investigator team consists of myself, Pablo Okhuysen who is the co-PI and Herbert DuPont. We have an excellent staff over at our clinical research center. If it weren't for these people, we'd have a terrible time trying to keep up with everything, so they really are essential. Madeline Jewell is the head nurse, and then we have two study nurses that basically are assigned to us, so we're very lucky. And we have a laboratory staff that has been very important in sitting behind the microscope for hours on end counting oocysts and doing all those other essential things that we base this study on.

We've gotten the isolates that I'll be talking about today from three sources. We got our IOWA isolate from Charles Sterling's laboratory in Arizona. And then the isolate that you've seen a couple of times today already, TAMU, was an isolate that was collected by Karen Snowden at Texas A&M University, which is where that acronym comes from. And then Joe Crabb had the UCP isolate at Immucell and shared that.
### Inclusion criteria

- Cryptosporidium serum IgM/IgG status
- Negative history and physical
  - Electrocardiogram
  - Chest X-ray
  - Stool for occult blood and parasites
  - Blood chemistry (SMAC)
  - Complete blood count
  - Urinalysis
  - Pregnancy test
  - Hepatitis BsAg
  - Hepatitis C
  - Immunoglobulin levels
  - Syphilis (RPR)
  - HIV
  - T-cell subsets

**Pass written examination documenting understanding of study.**

I want to tell you very quickly how we choose the volunteers and what we do before they're enrolled in the study. The very first thing that we do is to check them to see if they have serum antibodies to Cryptosporidium. We look for IgM and IgG antibodies. And then, depending on the experiment that we do, we either select those that are positive or those that are negative. And then we go through a long list of medical tests and evaluations. It was pointed out earlier in the talks that we only have studied healthy volunteers. You bet we only have healthy volunteers! We go to a big extent to make sure we only have healthy volunteers, because we certainly don't want to expose anyone to this organism that might be of increased risk of having a chronic or more severe disease. So we do go through a long list of things to make sure that they're quite healthy, and I would just point out one or two here. We not only look to see that they're healthy in other ways, but we check their immunoglobulin levels to make sure that they are able to make all antibody isotypes.

We want to make sure that we don't have any IgA-deficient people in this population. We have found one out of the about 300 that we've screened, so it's a wise thing to do. Of course we check their HIV status. That's very important. We also check their T cell subsets, not only for counts but for function.

So when we get through with this, they are deemed healthy if they have normal results on all these tests. And then we ask them to do one more thing, and that is to understand what we're telling them. If you are parents or you have been teachers or you have talked to large groups, you know that there is a difference in what you say and what's understood. And so we want to make sure that we're understood. After we go through our educational process with the volunteers, we ask them to take a test on all the major points that they should know about before they're enrolled. They have to make 100% on the test before they can go on with the study.
Exclusion criteria

- Infant under 2 years in household
- Elderly person in household
- Any contact with immunocompromised persons

We also have exclusion criteria that are very important. Remember, this is not a study where we lock these people up for six weeks in the hospital. They go home, they go to work, they go to school, they're active in their community, so we do want them to be very careful about secondary spread. And with that thought in mind, we exclude anybody who has an infant under two years of age in the household or who has elderly persons in the household or who might have any contact with any immunodeficient person or immunocompromised person.
Who have we enrolled in these studies? We're now up to 130 volunteers that we've studied, and this is a breakdown showing you the gender. I guess if this was a presidential election, that would be a pretty wide margin, but for our studies, the genders are about equal.

Ethnicity. We live in a cosmopolitan city and we've been very fortunate to be able to attract a number of ethnic groups to the study, so even though we have about 60 percent caucasians, we do have a good number of Hispanics, Asians, blacks, and a few Middle Easterners.

The age range; you understand that we're doing this in the Texas Medical Center, which has two graduate schools, two medical schools, a school of public health and 56,000 personnel that come and go every day, and so you might not be surprised to see that most of these volunteers are fairly young in age. A lot of them are students. Certainly, not all of them, but the majority are students. We enroll between 18 and 50 years of age.

You notice that there's one volunteer down here that's greater than 50. That's because he lied to us, and we didn't find out that he was actually 52 years old until we went to pay him and saw his Social Security information. But we try to limit it to 50 years of age. It's been suggested that elderly people might be more susceptible. I personally take offense at anything over 50 being called elderly, but my younger colleagues seem to think that that's a reasonable cut-off.
Let me tell you about the oocysts. All of the oocysts that we have used in these studies, the three that I'm going to talk about today that I listed for you earlier, are genotype 2s. They've all been passaged in calves in Doctor Sterling's laboratory at the University of Arizona. They purify them there. They send them to us in potassium dichromate. That, in part, is to protect the oocyst from any adventitious agents, that is bacteria or viruses.
Specifications for oocyst use

- **Negative microbiology (no viable adventitious agents)**
- **80+ excystation rate**
- **Within 6 weeks of passage**

Nevertheless, once they get to Houston, we immediately undergo two weeks of microbiological tests to be sure that they are free of adventitious agents. And, of course, that has to be negative before we'll then give them to the volunteers.

If those tests come back negative, then we adjust for the appropriate number that we wish to deliver that day. We instill those into gelatin capsules and we deliver the oocysts to the volunteers within an hour of their placement in the gelatin capsule.

So, the oocysts are used if they have negative microbiology, that is, no viable adventitious agents, if their excystation rate is greater than 80 percent, and if they are within six weeks of passage from the calf.
This is the study design that I’m going to show you today. I have gone through this first part of our collection and calf infection and purification. At the same time that we were dosing our volunteers, we also dosed the HCT-8 cell cultures, and Marilyn Marshall at the University of Arizona kept a portion of the same lot that we used in these other two studies and carried out dose-response curves in the CD-1 mice.
Again, just to very quickly go through this, since most of you are very aware of the history. The IOWA isolate and the UCP isolate were collected from calves with diarrhea, then passaged in calves a number of times over several years. In fact, in the laboratory, we know that these have maintained their virulence not only for the calves but for humans who accidentally got infected in those laboratories, so we know that over the years we've not lost that characteristic.

The TAMU isolate is a bit different. It is a "younger" isolate as far as laboratory passage is concerned. It was originally identified in a foal. They had an outbreak of cryptosporidiosis in a thoroughbred horse farm in Texas, and three foals were actually brought in. They all three succumbed to infection. This was the last foal, and it was only in that one that the organism was finally found. And then, when they went back, the other two foals were also positive on necropsy tissues.

During the necropsy there were three students that were assigned to the necropsy. They had not cared for the animal prior to that point. All three became infected. We got the oocysts from one of those students. It was in turn amplified in Arizona in a calf, and you will notice that all three of these isolates are stable, genotype 2 isolates by multi-locus analysis.
The volunteer study that we use is outlined here. We've talked about how they're selected. In this case, all of the volunteers were antibody-negative, they had no serologic evidence of having had this in the past. We challenged them with a single dose of *C. parvum* isolates, and then stool was collected every day for the first 14 days. And then we collected two 24-hour stools per week for a total experimental time of six weeks.

Now, we give them a small cooler that they carry over their shoulder that holds a collection kit so that they don't miss any opportunities. They're really very compliant, and we have good volunteers, so we've been able to get good data from them. At the same time that this is going on, they're also getting a daily physical examination at the clinical research center, they keep personal health diaries, including all the symptoms that they have. Those are audited daily by the nurses for accuracy and completeness. During the last four weeks, they are seen twice a week, so we have a very complete set of data on them.
Getting right to the infectivity. This is what we've found, and many of you have seen this, since it's already been published. Challenge dose and logs on the X axis, cumulative percent infection on the Y axis. You see here the IOWA isolate, which is the one that we started out with originally, gave us an ID 50 of 87. We went on to study the UCP isolate, and that isolate ended up giving us an ID 50 of about 1,042. And then we came back and studied the TAMU isolate --and I think you can appreciate the fact that that dramatically lower than the other two--and gave us an ID 50 of nine. We only went to a low value here of 10, because we didn't feel like we could accurately deliver fewer than 10 organisms.
The other thing that we saw was that there was a huge difference in the illness attack rate. What I've done here is try to simplify this into groups of people who have diarrhea. (We have very specific criteria for diarrhea.) And then those that didn't have diarrhea but that were shedding oocysts with or without symptoms. Some of these have milder symptoms that don't reach the level of criteria for diarrhea. And in this blue section are those individuals who did not have any oocyst detected and did not have any illness at all. So for all practical purposes, at least with our ability to detect infection, these people had no infection. That is, they have no evidence of infection.

And you can see that with the IOWA isolate you have about 52 percent of people that were exposed, who developed a diarrheal illness. About 59 percent in UCP--a little bit higher, even though the ID 50 was much higher in those people. But the real difference is with the TAMU, and that was almost 86 percent illness attack rate. You will also notice that there were no mild illnesses or asymptomatic infections with that particular isolate.
Now, the graph you see here illustrates the very same thing and shows you that there is a statistically significant difference in illness attack rate. But what I really want to draw your attention to in this slide is what was not significant. The incubation period, for example, was not significant. It ranged from five to nine days among the three isolates.

The duration of diarrhea was not significantly different--2.7 to 4.4 days of diarrhea and the total number of unformed stools. These both are measures of severity of disease. The total number of unformed stools ranged from 6.7 to 10.6 among the isolates, and the total unformed stool weight was not significantly different, although I point out here that TAMU did have the highest score. So there may be a trend to more diarrhea in these individuals, but it did not reach statistical significance.

Okay. So that's, in a nutshell, what we have found with the antibody-negative volunteers.
Let's turn our attention now to the same isolates tested at the same time in CD-1 neonatal mice. In this study, standard procedures were used. The mice were four to six days old, they were orally gavaged with a single dose of oocyst. They were then placed back with their dams until the termination of the experiment at seven days, and their infection was assessed by microscopy of the ileum.
These are the dose-response curves for the mice. Again you see that the TAMU isolate is the one that has the lowest ID 50. That was about 30 oocysts. And here, you see that the UCP and IOWA isolates are very close. They almost overlay each other. And the other thing you with notice is that the range of ID 50s here only goes from 30 to 99, where we were seeing 100-fold difference in the humans.
The HCT cell cultures were also studied at the same time that we were doing the volunteer experiments. In this case, the HCT-8 cells are placed in wells 18 to 24 hours before we infect them. That allows them to grow to about a 70 to 80 percent confluency. The oocysts are permeabilized with sodium hypochlorite. Some of this, as mentioned earlier, is because we had problems with contamination when we did not do this, so it is now standard procedure.

We add oocysts in a ratio of one to one with cells and the infection is developed for 24 hours. Then the cells are fixed and we count them after having stained them.
Cryptosporidium Infection in HCT-8 Cells

This is what a Giemsa stained cell culture looks like in our hands. Here are the cell nuclei, here's one in mitotic division. And then you simply count the number of parasitophorous vacuoles (foci) in this field. We also count the number of cell nuclei. We express the data as the percent of cells infected. And we count about 25 fields to get infectivity counts for each well.
This is the data that came from those studies. These are the three isolates that we've looked at in the volunteer and mouse studies. These are each done in triplicate for four different experiments, and so this is a standard deviation bar here. You can see that the TAMU isolate that had the lowest ID 50 has the highest infectivity in our cell culture, and it is significantly different from the other two. That's followed by the IOWA isolate that has an infectivity of about 37%, and then the UCP isolate was about 25%. So this is following the very same pattern that we saw in the human ID 50 curves.
If we take all of those data together and compare both the percent infection in the HCT-8 cells and the mouse ID 50s to the volunteer ID 50s, we can generate the following curves. You can see that when you impose a linear regression on this, the correlation with the HCT-8 results is very high. It's greater than .9.

However, when you look at the mouse infection, the mouse was unable to distinguish between the UCP and the IOWA isolates, so we didn't get very close data there. Again, the TAMU isolate is the one that is the most virulent, no matter what model system you're looking at, but the mouse data yielded an R-square value of 0.55.
Conclusions

- *C. parvum* oocysts vary widely in infectivity, even within the same genotype.
- HCT-8, a human enterocyte cell line, shows a higher correlation with human genotype 2 infectivity than does the neonatal mouse model.
- HCT-8 also has the advantage of supporting genotype 1 replication.

So the conclusions that we take away from these studies are that *C. parvum* oocysts vary widely in infectivity, even within the same genotype, so it's going to be very interesting to see what happens with the genotype 1s. HCT-8 cells, which are a human enterocyte cell line, shows a higher correlation with human genotype 2 infectivity than does the neonatal mouse model.

And just another plug for the HCT-8, because it also has the advantage of supporting the genotype 1 replication, and the mouse model does not. And so I'd be happy to entertain any questions that you might have. Yes sir, in the back.

AUDIENCE PARTICIPANT: I'm wondering if you got informed consent from the household members who might be exposed to oocyst shedding? And what about the long-term chance for reactivation in subjects if they take steroids for asthma or get HIV or develop cancer later?

Dr. CHAPPELL: Both of those are very interesting points. The first point was did we get informed consent from the household members. No, we did not, in a word. But I will follow that by saying that we have active surveillance of the household members and the household members were very well-informed of what was going on and what kinds of things to look for, so it's not that we ignore them. We just don't have to get informed consent. We also do an active surveillance of the household once a week. We call the contact person in the household. We always collect any diarrheic stool if they happen to occur and, as with the volunteers, anyone with diarrhea gets a complete microbiologic work-up to see if there is evidence of any other pathogen. (transcript continued next page)
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Transcript continued:

AUDIENCE PARTICIPANT: Has there been any secondary cases?

Dr. CHAPPELL: There has not been any secondary cases that we have confirmed. There is one probable secondary case in the German shepherd that one of the volunteers owned that had the habit of drinking out of the toilet bowl.

AUDIENCE PARTICIPANT: [Off mic.]

Dr. CHAPPELL: The second question, or the second part of that question was reactivation. We've been doing these studies since 1993. As you can imagine, it's not very easy to keep up with students and residents as they move around, but we do our best, and we have gotten anniversary sera from a number of them.

Actually, quite a good number. And at that time, we interviewed them for any points. We have not seen any evidence of any long-term problem or reactivation or any development of an inflammatory bowel-like syndrome or anything that has to do with a gastrointestinal infection. Yes sir?

AUDIENCE PARTICIPANT: [Off mic.] I had a couple of questions on the ? and I wondered if you saw a correlation between the degree of immune response, either antibody titer or by secreting cells or anything that correlated with the severity of the infection. First question. The second question is do you have any plans to go back to those volunteers and do an infection study by reinfecting them with the same or a different strain to see if they're protected or not?

Dr. CHAPPELL: Okay. The first question was about the antibody response, the immune response. What we have noted was a very strong correlation with secretory IgA, so those people that were infected had secretory IgA that could be detected in their feces. (transcript continued next page)
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Transcript continued: We did not see the same response in the serum. We all know that these are two distinct compartments of the immune system that are separately regulated.

And this answer really goes into the second question that you asked, and that is, have we done any rechallenge studies to come back and look at those individuals. Yes, we did.

With the very first group we invited them back for a one year anniversary party and were able to get 19 of them to come back. And we rechallenged them. You can imagine the difficulty in trying to figure out what study design to use, because each one of them had gotten different levels of inoculum the first time.

And so when they came back, we gave everybody 500 oocysts, which turned out to be about an ID 80. If I had it to do over again and knew what I know now, I would have upped it, but at that time we thought we were at ID 100, and we were not.

Nevertheless, we did see some evidence of protection, not in the fact that we prevented infections--the same percentage of people became infected, and the same percentage of people became ill--but what we did see was a dramatic decrease in the number of oocysts that they shed. So that has implications for secondary transmission.

The second thing we saw was that where we hadn't had any seroconversion in the first challenge, we had about one-third of them seroconvert after the second challenge, which leads us to surmise that when you go out and test this in the community, that the people that you're really calling antibody-positive that have evidence of infection may have had multiple infections in order to get the antibody titer up high enough for you to detect, or perhaps have a continuing
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AUDIENCE PARTICIPANT: [Off mic.]

Dr. CHAPPELL: Oh gosh, that's a good question. What the age distribution in asymptomatics in the three strains? You know, we haven't looked at that particular point, so I don't know.

AUDIENCE PARTICIPANT: [Off mic.] The CD-1 mouse.

Dr. CHAPPELL: How would I--how did we control spreading between the animals? The animals, those were done at the University of Arizona, and I don't know how many animals per cage, if they separated those one animal per cage or not, so I guess I would have to get back to you on that after talking with Marilyn Marshall about that point.

AUDIENCE PARTICIPANT: [Off mic.] Dr. CHAPPELL: Okay. So there are multiple animals in the same cage. It's a good point.

AUDIENCE PARTICIPANT: Do you have DNA samples archived for the volunteers if anyone wants to go back and look at the genetics in response to this?

Dr. CHAPPELL: Yes, for most of them. We didn't do that starting out, but we have done that, I think probably after about the first 15 or so. We do have samples now in the freezer. We have thought about doing HLA studies and so on, but it's a very expensive thing to do, and we don't have the funds yet to address that. Yes?

AUDIENCE PARTICIPANT: When you do this and you look at the fields and the cell counts, are the technicians blinded?

Dr. CHAPPELL: Yes. Thank you.