Hello and welcome. I'm Clare and you're listening to Microbe Talk, the podcast by the Microbiology Society. In previous episodes of Microbe Talk, we've covered the dangers of antimicrobial resistance, or AMR, and how resistant genes can spread in wastewater and the environment. AMR is a global health crisis, so it's vitally important that scientists are looking into new ways of tackling its spread.

So for this episode, I'm very excited to be speaking to David Walker-Sunderhauf about his recent paper published in Microbiology. David and his team harnessed the CRISPR-Cas gene editing system to directly target the sequences of DNA responsible for antimicrobial resistance, a method which shows exciting promise for combating the spread of AMR.

My name is David Walker-Sunderhauf. I'm a postdoctoral research fellow at the University of Exeter. I've been working on this particular project all the way through my PhD as well, so it's been nearly six, seven years in the making now.

Amazing. Amazing. So yeah, brings me on nicely. So the reason why we're on this call is because you published a paper recently in Microbiology, ‘The removal of AMR plasmids using a mobile broad host range CRISPR-Cas9 delivery tool’. So that's a lot of words that mean lots of different things. Could you kind of tell me about it? Like, what's the top on the bottom of your research?

Yeah, of course. So obviously, AMR, antimicrobial resistance is a huge issue. So bacteria are becoming resistant to antibiotics. And one of the problems is a lot of the times these genes, this DNA that makes them resistant to antibiotics is encoded on plasmids. Now, plasmids are mobile genetic elements, which means they can be passed on between different bacteria and different species.
And that’s how quite often we get antibiotic resistance genes passed between harmless environmental bacteria into human pathogens, which obviously can then nature cause issues in hospitals. So this is also a process that we can theoretically interfere with, which is why I’m looking at targeting these R plasmids, these antibiotic resistance plasmids, and removing them using a tool that we’ve developed here in the lab that uses CRISPR Cas9 to essentially cut DNA and remove these plasmids.

00:02:23:24 - 00:02:33:09
Clare Baker
Amazing. You sound amazing so, CRISPR-Cas9. This is quite a complicated kind of area. I mean, how does it work?

00:02:33:09 - 00:02:53:17
David Walker-Sunderhauf
So all it basically is, is a an enzyme that can cut DNA and it can cut a DNA, a very specific DNA sequence that you tell it to cut. Mm hmm. So CRISPR Cas9 in nature, it’s actually very naturally found in bacteria. It’s an immune system that bacteria use against bacteria, viruses which are called bacteriophage or even against plasmids.

00:02:53:19 - 00:03:14:19
David Walker-Sunderhauf
And in nature, basically, there’s two components to this parts. The Cas9 part is the enzyme, the nuclease that cuts DNA and the CRISPR part is the immune memory. So this what this does, it captures sequences of viruses that infect the bacterial cell. Mm hmm. And uses these short sequences later. If the same virus infects to cell again as a guide.

00:03:14:19 - 00:03:24:11
David Walker-Sunderhauf
So Cas9 uses the short DNA sequence as a guide to find the same virus and cut its DNA before it can do any harm. And if we want to use this for biotechnology, we basically just give it a synthetic guide.

00:03:24:13 - 00:03:25:13
Clare Baker
Yeah.
David Walker-Sunderhauf

Which means we can target any gene of our choice.

Clare Baker

So then what you’re doing is changing the CRISPR gene, as you said, the target to then target antimicrobial resistant bacteria and to microbial resistant genes, yet bacteria.

David Walker-Sunderhauf

Yeah, exactly. So we’re changing the the guide that Cas9 uses there, giving it a new target sequence, basically just we are giving it a predetermined immune memory. Okay. To target and cut antibiotic resistance genes.

Clare Baker

Okay. Amazing. And that only acts in the plasmid DNA. Does it affect the bacterial chromosome at all?

David Walker-Sunderhauf

It will cut all DNA that has the sequence. So you could theoretically use tools like this as well to directly target, say, pathogenic bacteria and cut their chromosome. And that would lead to death of these bacteria where the chromosome is cut in most cases.

Clare Baker

Wow. Interesting. So it can work potentially on two levels of removing antimicrobial resistance from bacterial microbial populations, but also removing them entirely and acting almost like an antibiotic itself.
Yes. Yes, absolutely. Although there's a whole range of more issues than that we need to think of. Obviously, if we’re trying to use this directly to kill bacteria, then the bacteria won't want to take up this CRISPR tool in the first place. Whereas if we’re just trying to kind of manipulate their genome a bit and remove any plasmids, any accessory genetic material that might be more amenable to taking up a tool like us.

00:04:53:16 - 00:04:56:10
Clare Baker
Sneaking in rather than getting in or guns blazing.

00:04:56:11 - 00:04:57:03
David Walker-Sunderhauf
Exactly.

00:04:57:04 - 00:05:03:21
Clare Baker
Okay, sure. So then, yeah, you're able to explain what plasmids are their role in antimicrobial resistance?

00:05:03:23 - 00:05:43:00
David Walker-Sunderhauf
Of course. So the way I like to imagine plasmids is they're just little loops of DNA hanging around inside bacteria, not part of the main chromosome, but huge, big jumbled message, genetic information that all bacteria have. But extra pieces of DNA that's basically hung around and they can be passed onto obviously vertically offspring bacteria, but also horizontally. So to other bacteria, even other bacterial species, even very unrelated bacterial species that might be found in the same environment, these plasmids can be exchanged, which means that any genes that sit on this plasmids will also be passed on to these other bacterial species.

00:05:43:02 - 00:06:13:07
David Walker-Sunderhauf
And because obviously we are using antibiotics so much nowadays in health care, in the environment, we find traces of antibiotics in the environment. There's always a benefit or quite often a benefit for bacteria to take up a plasmid that might have an antibiotic resistance gene on it. So that's like really commonly now we find one several, sometimes even dozens of antibiotic resistance genes sitting on a plasma that can be shuttled over to other bacterial species.
Clare Baker

So you have horizontal and vertical gene transmission within bacteria. Once these genes are cut and can no longer be present for antimicrobial resistance, that means that those genes can not be passed down vertically.

David Walker-Sunderhauf

Yeah, absolutely. So for cutting the antibiotic resistance genes, part of the plasmids, in most cases, this will lead to plasmid degradation, essentially. And there's no genetic material to pass on, either vertically or horizontally anymore.

Clare Baker

Okay. That's amazing. Okay, So I suppose in the general context of antimicrobial resistance is this silent pandemic. It's quite scary. And where does your research kind of fit in that context?

David Walker-Sunderhauf

So this is one of the many new approaches being investigated, obviously, and I think what's important about it is that we're not proposing that this is the solution and we don't need anything else. This is just one of the approaches that kind of work alongside other things. So as we said earlier as well, where what we're doing is we're removing resistance genes rather than killing bacteria directly.

David Walker-Sunderhauf

So obviously this means that we still, if we're trying to treat some sort of infection, for example, we do still need to apply antibiotics afterwards. So what this will do is, on the one hand, keep the antibiotics that we can that we already have. Keep them working for longer. And on the other hand, maybe interfere with transfer of antibiotic resistance, particularly in environmentally relevant compartments.
So in places like wastewater treatment plants and livestock farms, a lot of times we have a lot of exchange of genetic information between bacteria. And these are hotspots where we can find antibiotic resistance genes being passed from environmental bacteria to these human pathogens. And it's in places like this where an intervention like this could be really effective at just decreasing the overall load of antibiotic resistance that we have.

Clare Baker

Yeah, we actually just had Leonard on the podcast talking about wastewater and antimicrobial resistant monitoring, so listeners will know exactly the ins and outs of that. So this could potentially then be something that's like, I suppose, a step in the wastewater treatment process.

David Walker-Sunderhauf

Absolutely. Absolutely. Yeah. Yeah. To basically not necessarily remove pathogens or bacteria themselves from the from the wastewater, but to more remove these antibiotic resistance genes. So just make it less likely that problems in the future will arise through transfer of these genes.

Clare Baker

Mm hmm. Could it maybe, perhaps work like an individual level? Is it something that you could introduce to, say, for example, a person who's sick with MRSA? Could you treat them with this alongside antibiotics like you mentioned?

David Walker-Sunderhauf

Yeah. Yeah. So when we started off with the project, this was one of so there were these kind of two key application streams that we have in mind, one of them being this attack on these environmental reservoirs of antibiotic resistance. And the other one in patient care itself. So there the key thing is obviously, as we're removing the antibiotic resistance genes and we don't want to with this treatment any way kill bacteria directly, that we really want to time these this treatment.

David Walker-Sunderhauf

So the way we can kind of envisage is that if we know a patient will need antibiotics soon for example, if they've got a surgery scheduled or something that we can then first use on a CRISPR delivery tool such as this to clear any any of any antibiotic resistance genes that might already be
present in the patient, which means that then later when they get to undergo that course of antibiotics, we can be more confident that it will actually work.

00:09:49:02 - 00:10:05:07
Clare Baker
Wow. That's really interesting. So then, you know, on that level, you could say you'd be heading off anti-microbial resistant diseases, spreading in the hospital from the very start before they've gone into surgery, for example, before you're kind of cutting out offers had basically.

00:10:05:09 - 00:10:17:17
David Walker-Sunderhauf
Yeah. Yeah, exactly. So this is to me, one of the really exciting things about when about developing this basically that where it's the whole spread of resistance that can be interfered with.

00:10:17:19 - 00:10:31:19
Clare Baker
Yeah, that's really, really exciting. Really exciting. And you're targeting these antimicrobial resistance genes. Are they specific to specific antimicrobials or is it more general?

00:10:31:21 - 00:10:37:21
David Walker-Sunderhauf
Yeah. So what we're doing in this paper, in this study is targeting a very specific antibiotic resistance. You gentamicin resistance, Gene?

00:10:37:23 - 00:10:38:20
Clare Baker
Yeah.

00:10:38:22 - 00:11:11:07
David Walker-Sunderhauf
Not only is that specific to this antimicrobial, but it is very specific to the bacteria or to the versions of this gene that have exactly that nucleotide sequence that we we're targeting. So it's quite a specific treatment in that sense. However, what we are working on and what can be done in future is, for example, to use multiple different guides to give to give this CRISPR-Cas9 tool multiple
different targets so that it can cut several different versions of the same gene or several different genes even.

00:11:10 - 00:11:13:23

Clare Baker

Wow. That's so cool. And so and.

00:11:13:23 - 00:11:42:03

David Walker-Sunderhauf

Of course, yeah. And of course, if we're thinking about that, we're targeting antibiotic resistance plasmids themselves. So plasmids are really cool mobile genetic elements that carry more than just antibiotic resistance genes, of course, and sometimes can carry multiple antibiotic resistance genes. So we can start thinking about targeting a different region of this plasmid so that other backbone genes that this plasmid just needs to replicate itself that might be more conserved between different plasmas carrying different genes to make this whole thing a little bit more general.

00:11:42:05 - 00:11:51:19

Clare Baker

What what surprised you the most about your research in this paper? Is there anything that's kind of that came out like, you know, like, whoa, didn't expect that.

00:11:51:21 - 00:12:18:08

David Walker-Sunderhauf

Good question. I think the and it's not surprising in a sense that we this was one of the key things that we wanted to achieve with this study. But really cool that we can actually see it happening is that this CRISPR-Cas9 tool that we use, that we engineered, has a really broad host range. So we're actually using it in several different bacteria species in this paper and also in environmental isolates.

00:12:18:08 - 00:12:45:05

David Walker-Sunderhauf

So quite often when we're working, when we're working in the lab, everything is done in E.coli. We all know how it works. It's super nice and easy, but then sometimes you move into environmental isolates and you can't do anything to work anymore, or they just act differently. And here what we've done is we've made this really broad host range plasmids that encodes CRISPR-Cas9, and we actually use it directly in a series of both human associated isolates and isolates that come from from the environment.
David Walker-Sunderhauf

And in all of these, we can see that it can actually block uptake of antibiotic resistance.

Clare Baker

You use that term, isolate what do you mean by that?

David Walker-Sunderhauf

So, I mean, this is just where we get samples either from the patient or from the environment, some samples of example and or samples from pig P, for example. And and we just plate that out onto onto plates basically, and see what grows. And then we pick a colony and we isolated something from the environment.

Clare Baker

Okay.

David Walker-Sunderhauf

So this is, this is basically a bacterium which we know does exist in, in an environmental niche because that's where we got it.

Clare Baker

I see. So it's like proving almost like a real world example in the lab.

David Walker-Sunderhauf

Exactly. Because we get our model strain in this case from the real world.
Clare Baker

Yeah, I see. I see. Okay. And so actually, this is the kind of maybe breaking down a little bit, even just what the title means. Now. So you've talked about this broad host range. We've talked about CRISPR. It says like mobile in there as well. Why is that exciting?

David Walker-Sunderhauf

Yeah, so that's the CRISPR tool that we developed itself. So this is in itself a plasmid, a broad post range plasmid that we used and edited so that it can target other plasmids. And because of that, it's also got this mobile aspect of plasmid. So it can be passed between different bacteria. So that means the exciting thing is we have basically a delivery method of this perspective to our target bacteria that we could apply in the environment or in settings where we want it to work, because all we need is we need a donor bacterium that has this CRISPR-Cas9 delivery tool.

And because it can be passed on, because it can conjugate, it's mobile enough to then make its way into other bacteria that are present in the same environment.

Wow. Okay, so you've created this CRISPR-Cas9 tool. You created this plasmid as a way of tackling all the silent pandemic and in a way that's slightly different than what perhaps is. I suppose, what the public may think is, you know, perhaps maybe finding new antibiotics. Is it something completely different that right now right here? Like what what are your next steps in terms of this research?

Yeah. So in this paper, what we did is we basically used this plasmid, this CRISPR Cas9 tool against, I'd say against a quite synthetic lab plasmid that we have that carries an antibiotic resistance gene, have more as a proof of concept to say, yes, we have this target plasmid and we can remove it or we can block uptake of this plasmid.
But now what we're kind of doing is, on the one hand, moving to more clinically relevant antibiotic resistance. Genes carried, maybe carried on other plasmids, maybe carried even in chromosomes of bacteria, and trying to see how we can tackle them using the same tool. And on the other hand, as well, moving away from simple setups as we had here in this paper of one back to illustrate maybe two bacterial strains growing together to actually using this in bacterial communities.

So in a way, we've got several different species of bacteria grown together to kind of just add complexity onto this bit by bit and see how we need to optimize this to work in more complex settings.

Amazing. So I'm guessing then the way that it needs to wait is you're just adding complexity and adding more in the lab until you can prove that it works on an array of kind of methods.

Yeah, absolutely. But in that process, there are other things to take them out and maybe, maybe tweak how this CRISPR Cas9 tool works as well.

Yeah, Yeah. And then obviously there's these steps and then I guess there's quite a few more steps until it becomes something where, like you say, we're able to use it in wastewater treatment or we're able to use it on like a pharmaceutical level. There's elements of policy and there's elements of like different hoops maybe we have to jump through.

Is it quite far in the future then, to seeing this being used in the real world? What do you have to jump through? Basically, I guess is my question.
David Walker-Sunderhauf

Yeah, Yeah. So it’s a really, really interesting question. I’d love to have chats with anyone who knows more about this than I do. Basically, the key challenge is we kind of see in terms of policy, maybe regulation and ethics of using something like this, is that what we have is a, well, a plasmid that will pass around and spread anyway and does in the environment.

David Walker-Sunderhauf

But we’ve edited this to include CRISPR-Cas9 so that it can now target and remove other plasmids. And theoretically this could spread through our back to the community if we wanted to, of course. So that can remove antibiotic resistance genes, but it’s just sort of having the policy frameworks in place for doing this sort of genetic engineering. And I’d say it falls into a bit of a gray zone because we’re not actually editing any genes.

David Walker-Sunderhauf

What we’re doing is we’re removing genes that are already present and we’re not even removing species. So we’re not doing ecological engineering in that sense. We’re just changing the type, the types of genes that these species might be carrying.

Clare Baker

Mm hmm. I think that genetic engineering you said it and raise your eyebrows. It can be quite a scary time for a lot of people.

David Walker-Sunderhauf

Yeah, of course. The the other thing to remember with this is I mentioned that right in the beginning, CRISPR-Cas9 is a bacterial immune system. So about I think 50% of bacteria and archaea have a CRISPR immune system in nature. We you can find different types of CRISPR immune systems on plasmids as well. So mobile immune systems that go around and target different kinds of things, sequences.
So in a way, what this is, is not doing some sort of crazy genetic engineering, like introducing a whole new gene to plant or something to make it glow green. Yeah, but what we what we're doing is we're using a, an immune system that actually already used to usually target the viruses and reprogramming this to target antibiotic resistance genes instead.

Clare Baker

Yeah, exactly. Oh, gosh. Okay. I'm really interested. This is amazing. I'm so impressed. I'm people who are maybe not interested in science and not interested in microbiology. Why? Why should they be interested?

David Walker-Sunderhauf

Well, obviously, they're probably aware of or have heard the term of antibiotic resistance throwing around. We are so reliant on antibiotics nowadays, it's really difficult to understate how important it is that we actually tackling antibiotic resistance crisis. So.

David Walker-Sunderhauf

The thing is we're using these antibiotics for basically everything for routine surgeries, antibiotics are used so often once bacteria are resistant to all the antibiotics we have, we wouldn't be able to do things like routine surgeries. We wouldn't be able to treat a simple infection that you might get from having a simple cut. You have to go back to the time where you then lose your arm from having an infection so that you can survive.

David Walker-Sunderhauf

So it's really trying to tackle this issue before it's costing lots of lives.

Clare Baker
Yeah. One thing that scientists obviously love to do is to carry out their research. I'm not saying this yet, as.

00:19:57:24 - 00:19:59:06
David Walker-Sunderhauf
We need to be honest about it.

00:19:59:06 - 00:20:09:22
Clare Baker
AS Yeah, 100%. So we had some really exciting potential about your, your research. What, what is it not doing, What is I suppose the limitations at the moment.

00:20:09:23 - 00:20:38:00
David Walker-Sunderhauf
Yeah. So in this paper specifically, the main caveat I’d say is that there are, as I mentioned earlier, using a really synthetic target plasmid. So this basically is just a very small, very, very streamlined plasmid that carries an antibiotic resistance that much else, the sort of plasmids in nature that pass antibiotic resistance genes onto each other are far bigger, like at least ten times as big, sometimes 100 times as large as the plasmid that we're using.

00:20:38:06 - 00:20:54:01
David Walker-Sunderhauf
So they carry lots and lots of different genes. And these other genes that these natural plasmids can carry, they really sometimes promote parsons resistance. So they are genes that essentially make bacteria want to keep hold of the plasmids.

00:20:54:03 - 00:20:54:20
Clare Baker
Yeah.

00:20:54:22 - 00:21:06:15
David Walker-Sunderhauf
So therefore it's going to be a lot more difficult when we make these more of these larger, more relevant plasmid because they have all these other extra genetic material, all these other extra payload genes that might be interfering with this process of removal.

00:21:06:16 - 00:21:08:03
Clare Baker
Yeah, yeah, yeah.

00:21:08:07 - 00:21:31:02
David Walker-Sunderhauf
And the, the other caveats of is the magnitude of the fact that we're seeing. So this tool works really well in our paper and we're using as a barrier to uptake of antibiotic resistance genes. Mm hmm. We can also remove antibiotic resistance genes that are already present in a in a different at target strength using this, but it's a fairly modest effect that we're seeing.

00:21:31:02 - 00:21:56:12
David Walker-Sunderhauf
So I think it was something like a 20, 30% drop, maybe a bit more in target bacteria carrying an antibiotic resistance gene. Now, if it's just a small drop like that, it won't change things really in the in the longer term if we plan to, but it's afterwards. So the other caveat, what really we really need to do is optimize how efficiently this CRISPR plasmid gets delivered to the target bacteria.

00:21:56:14 - 00:22:22:14
Clare Baker
Yeah. Yeah. So fine tuning yet. Yeah. Do you foresee potentially I want to quote I won't be like lifelines away, but I really don't want to go like, what is that from like Jurassic Park or something? Yes, I think so. Without quoting Jurassic Park, do you foresee any way in which microbial communities could evolve a way in which to not be as affected by this genetically edited plasmid?

00:22:22:14 - 00:22:25:17
Clare Baker
Could that happen, or is it overtly targeted? Yeah, yeah, absolutely.

00:22:25:17 - 00:22:50:07
David Walker-Sunderhauf
So in the current form that we have it, that could absolutely happen. Again, I'm going to go back to CRISPR in nature as a bacterial immune system, just because we've had obviously millions of years of coevolution between bacteria with their CRISPR systems and bacterial viruses and bacteria, phage. So we know that in nature there are several different ways that bacteriophage can escape targeting by CRISPR Cas9 so they can evolve to overcome a process like this.

David Walker-Sunderhauf

And these are the kind of things we do expect to see with a treatment like this too. So one example would be that the target sequence of the antibiotic resistance gene that we're cutting, that it can just have some point mutation so that some of the nucleotides can change. But the gene is still functional. But this would mean that it's essentially invisible to CRISPR Cas9 right hand cuts anymore.

Clare Baker

Okay, so it's I can so specific that even a base change would mean that it.

David Walker-Sunderhauf

Yes. Yes, exactly. If the base changes in my position. Okay. Okay. But there are ways that we can potentially look at going around this. For example, using, as I think I've touched on earlier as well, if we use multiple different guides at the same time, we can only target several different genes, but maybe the same gene in several different regions, which would make an escape like this a lot less likely.

Clare Baker

I see. Okay. So it would be almost like a a longer sequence and you're targeting multiple specific areas within that.

David Walker-Sunderhauf

Exactly. Because then for the gene to completely escape the targeting process, it would have to involve a mutation like that in several different spots at the same time.
Clare Baker

Yeah. Yeah. Okay. And so there's so much talk about creating good microbes back microbes and microbiome and how great that is in the gut, free access and all of this amazing stuff about how microbes can be really good for you. They get a little bit of a bad rep. Is there potentially a situation in which this could affect kind of microbial communities in your body and have negative effects?

00:24:20:06 - 00:24:24:07

Clare Baker

Is that is that some not keeping down is on all of this amazing research? Yeah.

00:24:24:07 - 00:24:57:12

David Walker-Sunderhauf

And of course if it's good it's really good to talk about these things. And that's one of the really cool things about this approach is I think because firstly we're so specific in what we're targeting because CRISPR-Cas9 so specific, we can be absolutely certain that it's just microbes that actually carry this gene sequence of interest. So in our case, antibiotic resistance genes that are affected by the treatment and and on the other hand as well, because we're targeting mobile antibiotic resistance genes, we're targeting the plasmids, we're just changing the genes that these microbes are carrying.

00:24:57:12 - 00:25:17:05

David Walker-Sunderhauf

The microbes will still be there. They'll still be part of the community. And in many cases, it might even turn back and make this into a good microbe, so to say, because quite often we have virulence genes that are also carried on these plasmids, and it's just plasmids with armor genes, plasmids with virulence genes that are turning these good microbes into bad microbes.

00:25:17:07 - 00:25:22:21

David Walker-Sunderhauf

So what we're doing is we're essentially changing the changing the content of genes on the same.

00:25:22:23 - 00:25:44:23

Clare Baker

And this could be really useful as well in terms of if you're taking antibiotics, affects your gut microbiota, right? So it means that it could potentially alter the balance of microbes in your gut and make sure that A Well, so in taking this, it's targeting, like you said, the microbial community doesn't change with antimicrobial resistance genes in that community changes.
So it could be really, really healthy for your gut, for your lungs, and for the microbial communities in your body.

Yeah. Yeah, exactly. Although if we're thinking about applying to some patients that the idea would be to use something like this and venues antibiotics. Yeah. So in those cases we wouldn't, we wouldn't get around these issues. But in general, if we use an approach like this and maybe optimize it for a situation where we know there's a specific plasmid just turning a bacterial species into a bad microbe, so to say we could, instead of using this and target antibiotic resistance genes to clear this virulence possible.

Amazing. Okay. And I, I have absolutely loved speaking to you. I think this paper is so interesting. If listeners would like to read more about your research and that is 'The removal of AMR plasmids using a mobile broad based range CRISPR-Cas9 delivery tool' published in Microbiology. If they want to find out more about your research and follow you going forward, do you have like a LinkedIn or?

Yeah, it's probably easiest on Twitter. I'm quite active on Twitter and scientifically. So if you follow me on Twitter, my handle is @davvi36 or can also send me a message, on there, happy to get back to people with any questions as well. Yeah.

That's fantastic. Okay, I will link that in the description of this podcast, but yeah, thank you so much. It's been such a pleasure.
Thanks so much for inviting me on this. Yeah, it was great to speak to you.

00:27:19:05 - 00:27:29:14

Clare Baker

You've been listening to Microbe Talk. If you want to read David's full article, you can find it linked in the description below. And if you like this episode, please leave a comment or like wherever you're listening.