Cantaurus, Vol. 23, 16-20, May 2015 © McPherson College Department of Natural Science

Is *Clostridium perfringens* present in soil samples from central Kansas?

Sydney Lipton

ABSTRACT

Clostridium perfringens has been isolated from soil samples located in central Kansas. *Clostridium perfringens* is a spore forming anaerobe that can produce up to four toxins. These toxins range in risk factors but all can be very dangerous. The identification of *C. perfringens* can lead to the proper precautions that need to be taken when being exposed. While *C. perfringens* type A is known to be present in humans digestive tract, type B has recently been isolated from a human for the first time. Therefore, it is important to know and understand the means of possible exposures. Four locations in Kansas were tested, along with a stool sample from a female participant. These locations include: Haysville, Wellington, Wichita, and McPherson. From the four locations, only the Haysville sample did not contain *C. perfringens*. Selective agar for *C. perfringens* was used to determine this given that the bacteria colonies grow a certain color. The feces sample also contained C. perfringens, which was expected.

Keywords: *Clostridium perfringens, Epsilon toxin, ruminant*

INTRODUCTION

Clostridium perfringens is a rod-shaped, Gram positive bacterium. This spore forming anaerobe is ubiquitous in nature and found as a normal component of decaying vegetation, marine sediment, the intestinal tract of humans and other vertebrates, insects, and soil. It is commonly present in intestinal tracks, feces, water, and soil.

There are five toxinotypes (A-E) of *Clostridium perfringens*. They are defined based on the type of toxin they produced. These toxins are alpha (α), beta (β), epsilon (ε), and iota (ι). The α-toxin is produced by all toxinotypes, the β-toxin is produced by types B and C, ε-toxin is produced by types B and D, and the ι-toxin is produced by type E (Hadimli, et al., 2012). These exotoxins play a crucial role in disease pathogenesis which include gas gangrene, enteritis necroticans, and food poisoning.

This bacterial species is responsible for nearly one million illnesses each year and for most of the foodborne illnesses in the United States. *Clostridium perfringens* releases a toxin when inside the small intestine that leads to diarrhea or severe gastroenteritis. This in turn can cause damage to the small intestine and sometimes lead to death.

The bacteria can be transmitted to humans through ingestion or open wounds through contact with contaminated food, water, and soil. *Clostridium perfringens* food poisoning outbreaks are usually caused by contaminated beef, poultry, gravies, and dried or precooked foods. *Clostridium perfringens* is also found in animal intestines and feces. Type B thrives in the intestines of ruminant animals that eat a carbohydrate-rich diet. These ruminants include domestic cattle, bison, buffalo, camels and llamas, giraffes, deer, pronghorn, antelope, sheep, and goats. *C. perfringens* have even been isolated from a horse's intestine. Of these animals, cattle, deer, and sheep can be found in Kansas.

Therefore, if these animals are contagious they are contaminating the soil that humans come in contact with. Even if people are not coming in contact with the soil the animals are currently living on, animals may have lived in areas that are now occupied by humans. Because *C. perfringens* creates spores to survive in unfavorable conditions, these bacteria could still be lurking in the soil. These bacteria have also been recently found as a possible environmental trigger to the disease; Multiple Sclerosis.

This bacterium causes a health concern to humans. However, there is more research on *C. perfringens* infections due to contaminated food than there is due to contaminated soil. Since this bacterium is also found in nature, more research should be done to test the prevalence of it in the soil. Knowing what areas in nature that are likely to be contaminated will aid in further research on the epidemiology of this bacteria (Rumah, et al., 2013).

MATERIALS AND METHODS

Because *Clostridium perfringens* is found in the environment, the goal of this research is to determine if this bacterium is present in the soil. Also, to aid in the research a person's fecal specimen was tested to confirm whether or not *Clostridium perfringens* is present in human intestines.

The fecal sample was self-collected from a 25 year old female MS patient. A fraction of the stool sample was analyzed in the lab to determine what types of bacteria were present.

Sample Collection Process

The stool sample was self-collected by the patient in a clean single use container and stored in a cooled portable cooler to preserve it while returned to the lab. Approximately one gram of the sample was stored in

a collection tube containing 9 ml of buffered glycerin-salt (10% glycerin, 71.2 mM K_2HPO_4, 29.4 mM KH_2PO_4, 71.9 mM NaCl made in distilled water, adjusted to pH 7.2 and autoclaved) (Rumah, Linden, Fischetti, Vartanian 2013). The samples were re-suspended in 40 ml of modified rapid perfringens media (RPM) and cultured in 50 ml falcon tubes with tightly closed caps at 47 ˚C (Rumah, Linden, Fischetti, and Vartanian, 2013).

Since *Clostridium perfringens* is found in the environment due to the fecal contamination by ruminant animals, collecting and analyzing soil samples determine where this bacterium is present. I gathered samples from central Kansas; Wellington, Wichita, Haysville, and McPherson. The purpose of analyzing soil from these locations is to determine if there is a possibility that the MS patient was exposed at one of these areas.

Soil samples were collected from public parks at each location with sterile falcon tubes. The soil samples were collected off the top soil layer from different areas of the parks. In Wellington, soil samples were also collected from a residential property. This is where the MS patient lived as a child. The property has a variety of animals living on it, including horses. At one point, cows lived here. So, from that location, soil was taken from the pasture, corral, and barn stalls. A total of 11 soil samples were collected.

Isolation and Characterization of *C. perfringens*

Approximately 0.5g of the patient's fecal sample was suspended in 10 ml of phosphate buffered saline (PBS) [K_2HPO_4 3 g/ml, KH_2PO_4 1 g/ml, NaCl 8.5 g/ml; (pH 7.2 ± 0.2)] + 0.1% Tween 80 (Rumah, Linden, Fischetti, and Vartanian, 2013). Of this, 5 ml was heat-shocked at 85 ˚C for 15 minutes before inoculation into CP ChromoSelect Agar (Rumah, Linden, Fischetti, and Vartanian 2013). This promotes spore formation and inactivates vegetative bacteria. These cultures were incubated overnight at 37 ˚C and the next day, 100 µl of the fecal cultures were inoculated onto CP ChromoSelect Agar that is selective of *C. perfringens*. Green colonies are characteristic of *C. perfringens* on this media. To isolate pure colonies, subcultures were streaked onto CP ChromoSelect agar again.

From each soil sample, 1g of soil was suspended in 10 ml of sterile phosphate buffered saline (PBS). The samples were then heat-shocked at 85 ˚C for 20 minutes and cooled at 44 ˚C. Heat-shocking inactivates the vegetative bacteria and enhances sporulation of *C. perfringens*. Next, the samples were inoculated onto CP ChromoSelect Agar. After the solution was absorbed into the agar, another thin layer of agar was poured on top. This creates an anaerobic environment for the *C. perfringens* to grow in. Green colonies of anaerobic *C. perfringens* bacteria should appear. Again, subcultures are streaked onto ChromoSelect Agar in order to isolate pure colonies.

RESULTS

Identification of *Clostridium perfringens*

Identifying started with the inoculation of the suspended samples onto CP ChromoSelect Agar. The plates were inoculated by pipetting 100 µl of the suspended solutions. The solutions were then smeared on the plate. The presence of *C. perfringens* was determined by the growth of green colonies.

Table 1. Identification of *C. perfringens* from green colonies. The "x" in the cells under the "yes" column indicates that *C. perfringens* did grow on the CP select Agar.

CP ChromoSelect Agar + T.S.C. Supplement		
Fecal Samples		
	Yes	No
1	X	
2	X	
3	X	

There were three plates of inoculated fecal matter. Each plate was covered in bacterial growth. There were smears of black and grey colors on the plate. The presence of green in the plate determines the growth of *C. perfringens*. Each plate had substantial growth.

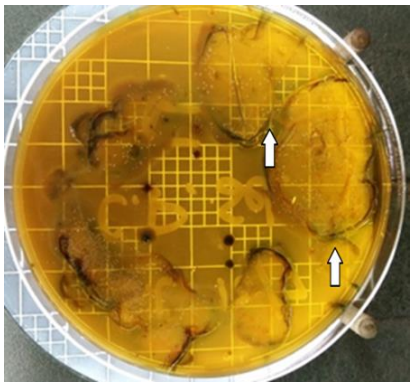


Figure 1. Growth of *C. perfringens* from human feces samples. Foggy areas (indicated by the white arrows) around the black areas are green and indicate the presence of *C. perfringens*

Next were the soil samples. The same process was used to inoculate the plates as with the fecal samples. 100 µl of the suspended samples were inoculated onto the CP ChromoSelect Agar.

Table 2. Identification of *C. perfringens* from green colonies. The “x” is the indicator if *C. perfringens* grew

CP ChromoSelect Agar + T.S.C. Supplement			
Soil Samples			
Location	Sample	Target Colonies	
		Yes	No
Haysville	1		x
McPherson	1		x
	2	x	
Wellington	1		x
	2		x
	3	x	
	4		x
Wichita	1		x
	2		x
	3		x
	4	x	

Figures 2, 3, 4, and 5 show the growth of Clostrium perfringens on CPChromoSelct Agar plates. Some plates had no growth of anything and most had black complexes.

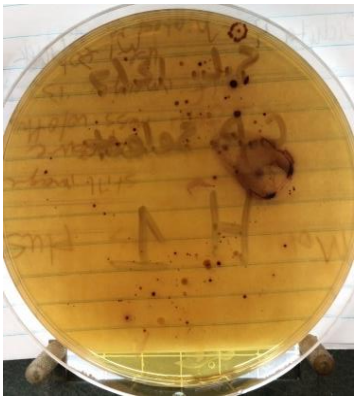


Figure 2. This plate contains the soil sample from Haysville, KS. Black complexes can be seen but are not identified.

After the growth of colonies, samples of the colonies were taken from the plates and put onto microscope slides. Three slides were prepared, which included the McPherson bacteria colony, Wellington bacteria colony, and the Wichita bacteria colony. Photomicro- graphs of these three slides are shown in Figures 6, 7, and 8.

Six out of the 12 samples (including the feces) had *Clostridium perfringens* colony growth. All three of the stool samples had *C. perfringens* growth. Figure 1

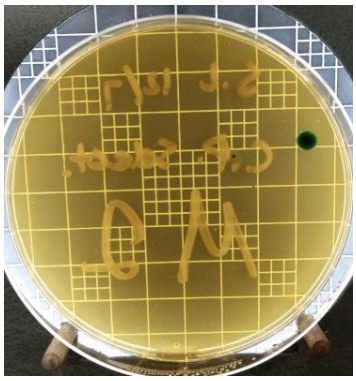


Figure 3. One large green colony of *C. perfringens* is present on this plate. This plate holds the soil from McPherson, KS

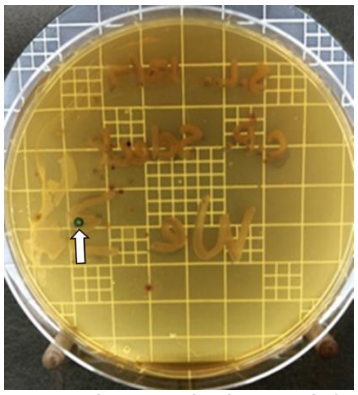


Figure 4. Green colony on the bottom left side indicates *C. perfringens*. This sample is from Wellington, KS.

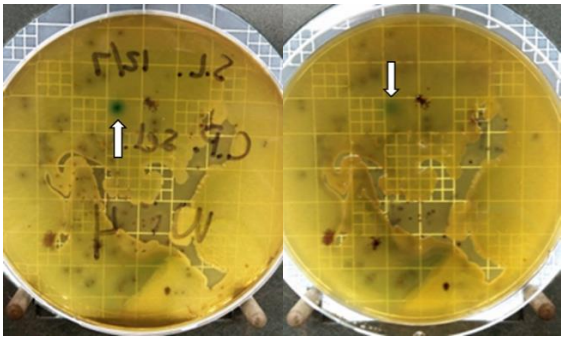


Figure 5. This plate holds the soil sample from Wichita, KS. The green colonies deep in the agar indicate growth of *C. perfringens*.

shows the growth on one of the plates that contained fecal matter. All three plates of the fecal sample were covered with bacterial growth. The areas around the dark marks are green, which indicate the excessive growth of *C. perfringens*. The plates had no individual colonies of *C. perfringens*, but they were covered in green material; on top, within, and under the agar. This meant there was a high concentration of *C. perfringens*. The Haysville sample, as shown in Figure

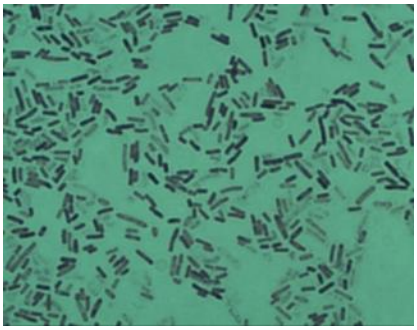
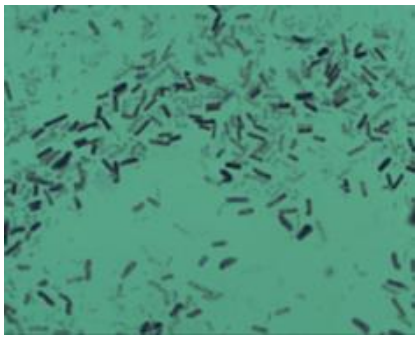
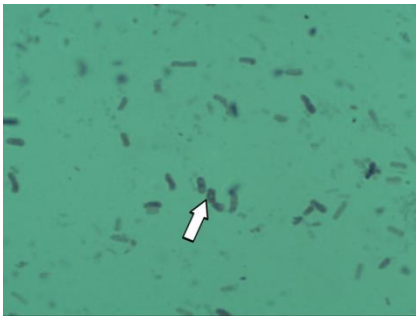


Figure 6. Image of *Clostridium perfringens* through a light microscope from the McPherson park soil sample.



a.)



b.)

Figure 7. (a) Bacteria from Wellington, KS. (b) *C. perfringens* that has begun to produce endospores (indicated by the white arrow).

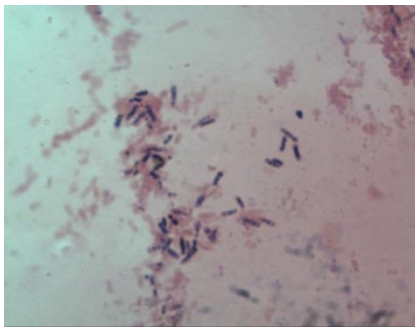


Figure 8. *C. perfringens* from the colony growth of the Wichita community park sample.

2, had no green colonies grow, but it did have multiple black complexes. In fact, most of the plates had these marks on them.

Only one sample from McPherson had *C. perfringens* growth. Figure 3 shows the growth of one fairly large green colony that was produced from the McPherson sample. Figure 6 confirms the growth of *C. perfringens*. Similarly, one sample from Wellington had a single green colony grow on it. This is seen in Figure 4 and confirmed when observed under the microscope, as in Figure 7. The green colonies on these two plates were on the top of the agar, while the green colony growth on the Wichita sample was under the agar on the bottom of the plate. Figure 5 shows the top and bottom of the agar plate. The green colony on this plate is within the agar. This is significant in determining that it is an aerobic bacteria that grew and because it is green, it determines that it is *C. perfringens*. Figure 8 also confirms this.

DISCUSSION

The survey did detect *Clostridium perfringens* in the soil samples that were collected from different areas in Kansas. From the 11 soil samples analyzed, three contained *C. perfringens*. The CP ChromoSelect Agar plates that were found with C. perfringen growth were those that had green colonies growing on them. The green color is a characteristic of the detection *of C. perfringens* growth, which is why this selective agar was used. It was no surprise that the stool samples also contained *C. perfringens*. This bacterium is found in the digestive tract of humans.

Every sample had black complexes grow. Based on the information given in the Sigma-Aldrich kit used to make the agar plates, the black complexes are probably the presence of iron. The Haysville sample only had the black complexes; no *C. perfringens* growth. This was surprising because that sample came from an area where livestock occasionally passes through. However, this could be due to the time of year the sample was taken. It was in the fall when the weather was cold and the animals were not passing through anymore. The sample was only taken from the top layer which means if the bacterium is there due to contamination by animals, it could be deeper within the soil.

Two samples from McPherson were taken and tested. They were collected from the same park but from different areas in the park. The sample that had *C. perfringens* growth was from the soil in a play area. Four samples were taken from Wichita, each one from a different park or public area. The sample that had growth was from a community park. The soil was taken from the edge of the pond that is part of the park. This is the sample that had the colony grow in the agar at the bottom of the plate (Figure 5). One sample from Wichita that did not have *C. perfringens* was from a park area beside a hospital. This park area is specifically for people that are in the hospital. Nurses take smoke breaks out there and visitors rest there.

One would think that the trafficking of nurses and visitors from inside the hospital would lead to a contamination in the soil. Perhaps the hospital does an excellent job in sanitizing.

Four samples were also taken from Wellington. The sample that had *C. perfringens* grow was from a corral on a farm. This corral is occupied by horses. *C. perfringens* type A has been isolated from the intestines of healthy horses and so it makes since that this ample would have *C. perfringens* present.

It is determined that *Clostridium perfringens* is present in soil samples. These soil samples came from public areas where humans and animals interact. This research can be added to the other research on *Clostridium perfringens*. While bacterial food poisoning is well researched, now there is more research on other means of exposure. Perhaps people that catch the “24 hour bug” actually came in contact with *Clostridium perfringens* from being outside and exposed to soil that has the bacteria residing in it. People now may want to make sure their cuts are bandaged before exposing themselves to soil and will want to wash their hands afterwards. This research leads to the conclusion that people could become exposed to *Clostridium perfringens* through contact with soil. Better sanitary precautions should be taken when coming in contact with soil outside.

ACKNOWLEDGEMENTS

I would like to thank my advisor Dr. Frye. His support and direction has aided me every step of the way. I would also like to thank the participant who provided the stool sample in order for my research to be well rounded.

LITERATURE CITED

Hadimli, H, O Erganiş, Z Sayin, and Z Aras. 2012. Toxinotyping of Clostridium perfringens isolates by ELISA and PCR from lambs suspected of enterotoxemia. Turkish Journal of Veterinary and Animal Sciences, 36(4), 409-415.

Marcheggiani S, M Iaconelli, A D-angelo, E Pierdominici, G La Rosa, M Muscillo, M Equestre, and L Mancini. 2008 Microbiological and 16S rRNA analysis of sulphite-reducing clostridia from river sediments in central Italy. BMC Microbiology, 81-11. doi:10.1186/1471-2180-8-171

Popoff M. 2011 Epsilon toxin: a fascinating pore-forming toxin. FEBS Journal 278(23):4602-4615.

Rumah KR, J Linden, VA Fischetti, and T Vartanian. 2013. Isolation of *Clostridium perfringens* Type B in an Individual at First Clinical Presentation of Multiple Sclerosis Provides Clues for Environmental Triggers of the Disease. PLoS ONE 8(10): e76359. doi:10.1371/journal.pone.0076359

Sayeed S, J Li, and BA McClane. 2010. Characterization of Virulence Plasmid Diversity among *Clostridium perfringens* Type B Isolates; 78(1):495–504. doi: 10.1128/IAI.00838-09

Schoster A, LG Arroyo, HR Staempfli, PE Shewen, and JS Weese. 2012 Presence and molecular characterization of *Clostridium difficile* and *Clostridium perfringens* in intestinal compartments of healthy horses. 6: 91, doi: 10.1186/1756-0500-6-9.

2014 Clostridium perfringens. Centers for Disease Control and Prevention. Web. 27 Mar. 2015. <http://www.cdc.gov/foodsafety/clostridium-perfingens.html>.

"Clostridium perfringens." Clostridium perfringens. U.S. Department of Health & Human Services. Web. 27 Mar. 2015. <http://www.foodsafety.gov/poisoning/causes/bacteriaviruses/cperfringens/>.

Clostridium perfringens Food Poisoning (Gastroenteritis: Merck Manual Home Edition) Web. 29 Mar. 2015. <http://www.merckmanuals.com/home/digestive_disorders/gastroenteritis/clostridium_perfringens