STEC Derived HUS: Infection of Toxemia?

The meat industry worldwide seems currently under attack by a bacterium we know well as a generally non-trouble-making resident of the human colon (*Escherichia coli*), seemingly lately possessed of a terrible "new" weapon in form of Shiga toxin. We call this beast "Shiga toxin *E. coli*" (STEC). We wonder where it came from and why it was sent to plague us.

However, *E. coli* has its problems too. It serves as prey to a number of viruses (bacteriophage). Most bacteriophage use the cell of the *E. coli* to make new virus particles, killing the infected bacterium in the process. There is another, much more sinister, pattern of bacteriophage infection termed a "lysogenic" cycle. Once inside the host cell, the lysogenic viral DNA has a choice to make. It can proceed directly to a lytic cycle to make more bacteriophage or it can integrate itself within the chromosome of the host and stay there, mostly but not quite senescent, dividing whenever the bacterial chromosome divides. The genes that code for Shiga toxin reside, not on the bacterial chromosome itself but rather as part of the lysogenic viral genome. When lysogeny is broken and the lytic cycle ensues, Shiga toxin is made along with other late stage products such as the proteins (capsids) needed to make the bacteriophage case.

To see a video of bacteriophage attack upon a host bacterial cell see:
http://www.nsf.gov/news/news_videos.jsp?org=NSF&cntn_id=100420&media_id=51295

To see the lysogenic and lytic cycles compared see:
http://highered.mcgraw-hill.com/sites/007352543x/student_view0/chapter20/lambda_phage_replication_cycle.html

Most (but not all bacteria) are quite tiny. For example a typical *E. coli* cell growing on glucose minimal medium with a doubling time of 40 minutes is estimated to have a wet weight of 9.5×10^{-13} grams [Ingraham, J. L., O. Maaloe and F, C, Neidhardt, 1983, "Growth of the Bacterial Cell", Sinauer Associates, Inc., Sunderland, MA, p. 3]. Nothing says this number can't be rewritten in the form of 0.95×10^{-12}. Furthermore, we can (because it is true) claim that the fraction 0.95 is very close to one. That way we can say that the wet weight of our average bacterial cell is about 1×10^{-12}, which just happens to be a fraction of a gram we define to be a picogram (pg), or one trillionth of a gram.

This discussion of the relative sizes of things illustrates a very powerful reason for why people may prefer to measure the prevalence of STEC rather than the toxin carried by STEC. If one extracts all the bacteria from a gram of sample and finds that such a sample yields a single colony of STEC, then one has analyzed at a very high analytical sensitivity indeed. The single bacterium that gave rise to the colony identified as STEC weighed one picogram. By definition, a gram contains a trillion picograms. In this example, the analyst has determined that one-trillionth part of the gram of sample was a dangerous bacterium. Without going into rather complex sampling issues, it is nevertheless true that such a result could be arrived at using relatively simple and inexpensive tools with a theoretical analytical sensitivity in the area of parts per trillion. By comparison, a

chemical or biochemical test run at an analytical sensitivity of parts per trillion usually involves a roomful of equipment that is expensive to acquire, expensive to maintain and operated by highly-trained personnel who want not just a good salary, health care, vacations and coffee breaks, but who also want to be loved.

A ribosome is a complex molecular machine whose job it is to make new proteins. Ribosomes have two components, one of which is larger than the other. Ribosomes also come in at least two flavors, eukaryotic and prokaryotic, which have a similar function but differ in the details of how they work. Eukaryotes are cells possessed of a "true" nucleus, surrounded by a nuclear membrane (like humans, for example). Prokaryotes (like bacteria) seem to get along just fine without a true nucleus. The large and the small ribosomal subunits (of both prokaryotes and eukaryotes) are an integrated configuration of ribosomal RNA and ribosomal proteins. A messenger ribonucleic acid (mRNA) is a transcript of the genetic (DNA) information needed to specify the amino acid sequence of a given protein a ribosome is asked to manufacture. This messenger RNA is held between the small and large ribosomal components. As each amino acid is added to the growing polypeptide chain, the messenger RNA must move (relative to the ribosome) precisely one codon (nucleotide triplet) in order for the next amino acid in the sequence to be specified. This movement of the messenger RNA relative to the ribosome is termed translocation. This process of DNA-directed, RNA transcript mediated protein synthesis on a ribosome is central to life (at least on this planet). This process demands much from the cell, in terms of both energy and infrastructure investment. In return, it helps to make life possible.

Shiga toxin is made up of two components. One of these (subunit A) is an enzyme (N-glycosidase), which is able to remove certain critical pieces from the ribosomal RNA of the eukaryotic large ribosomal component. This reaction results in a large ribosomal subunit that is defective. It is no longer able to bind the elements that make translocation possible. No translocation, no protein synthesis. The infrastructure investment the cell has made in manufacturing that ribosome is lost. The energy the cell has set aside to run protein synthesis on that ribosome now has nowhere to go. From the point of view of the Shiga toxin enzyme, is has just converted its substrate (a functional ribosome) into a product (a broken ribosome that is now out of the protein synthesis business). Nothing left to do but find all the other substrates (*i.e.,* working ribosomes) in that cell and convert them to product (*i.e.,* broken ribosomes) as well.

When a cell can no longer synthesize protein, it dies and then disintegrates, releasing the Shiga toxin. This is when the other end of the Shiga toxin comes into play. The B subunits, usually arranged as a pentamer, are there to bind to the cellular membrane of the next target cell and to permit the Shiga toxin to penetrate that membrane through endocytosis, an invagination of the host cell membrane. If that invaginated vesicle were to fuse with a lysosome the Shiga toxin would be destroyed. However, if the invaginated vesicle were to interact with the cell's Golgi apparatus, then the Shiga toxin will be released into the cell's cytoplasm where it can, again, destroy ribosomes.

The entire Shiga-toxin complex has a weight of roughly 70,000 daltons. This means that one molecule of Shiga toxin has a "standard" weight (*i.e.,* in grams) of about 116.238 zeptograms. A zeptogram is one sixtillionth of a gram (1×10^{-21} grams). So, a billion zeptograms is equivalent to one picogram (1×10^{-12}). A million picograms equals one microgram (1×10^{-6}), you may visualize as the average weight of one average human fingerprint. Why all this numerology in the first place? So we can count molecules. For example, one nanogram is a billionth of a gram (1×10^{-9}). The foregoing numerology allows us to calculate that one nanogram of Shiga toxin represents roughly 8.6 billion molecules of toxin. We already know that we only need 10 to 100 STEC cells to cause enterohemorraghic colitis and hemolytic uraemic syndrome.

Given what we know, how can we envision coming by 8.6 billion molecules of Shiga toxin from ten to one hundred cells carrying the plasmid? Well, we know that STEC, like all E. coli, are adapted to life in the colon and will grow there with a doubling time of 40 to 80 minutes so long as these cells are able to insinuate themselves into the intestinal mucosa [Poulsen, L. K., T. R. Licht, C. Rang, K. A. Krogfelt and S. Molin (1995) "Physiological state of Escherichia coli BJ4 growing in the large intestine of streptomycin-treated mice", J. Bacteriol. **177**(20):5840-5845]. Using the slower growth rate (80 min.) and the lower estimate of infectious dose (10 cells), just to be on the safe side, we see that if we allow 10 individual STEC bacteria to complete thirteen successive cell divisions in the intestine that there will be 81,920 bacterial cells, a relatively small number as bacterial infestation goes. Remember that one cells weighs about one picogram. That means that 81,920 cells will weigh 81.92 nanograms or 0.08192 micrograms – less than one tenth the weight of the average human fingerprint. The time it takes for this buildup of bacterial Shiga toxin carriers is (assuming the slowest reported growth rate) about 1,040 minutes or a little over 17 hours. We already have good reason to believe that the Shiga toxin is a late-gene product of the lytic cycle. For example, [Plinkett, III, G., D. J. Rose, T. J. Durfee and F. R. Blattner (1999) "Sequence of Shiga toxin 2 phage 933W from Escherichia coli O157:H7L Shiga toxin as a phage late-gene product," J. Bacteriol. **181**(6):1767-1778] where " . . . toxin production may be coupled with, if not dependent upon, phage release during lytic growth. So, if an event were to take place that resulted in the termination of the lysogeny of the Shiga toxin carrying prophage, then we may assume that the virus carrying Shiga toxin will enter the lytic state, causing the construction of at least 150 new viruses/cell, a conservative estimate. In the course of the lytic cycle, virus proteins will be made at a very rapid clip. A further assumption is that for each new virus constructed, about 1,000 late-stage viral proteins (including Shiga toxin) will be made by the doomed *E. coli*. So, $81{,}920 \times 150 \times 1000 = 1.2288 \times 10^{10}$ Shiga toxin molecules (*i.e.,* 12.3 billion) will be made, way more than the 8.6 billion molecules whose provenance we need to explain.

Several investigators engaged in the study of STEC have remarked that they did not find this organism to be particularly invasive. This finding is consistent with the notion that enterohemorraghic colitis and hemolytic uremic syndrome is the result of the toxicant and not of the "pathogen" carrying it.

The premise that STEC is not so much a pathogen as a carrier for a very potent toxin and that therefore enterohemorrhagic colitis and hemolytic uremic syndrome are not so much examples of an infectious disease as they are of a potent colonic toxicosis (which develops later into a toxemia) would be more convincing if evidence were in hand, which showed that these disease states could be attained without the detectable presence of STEC at all. Such evidence exists. For example, the first instance of an *E. coli* O157 outbreak in Denmark was actually attributed to milk coming from a dairy where the milk had been pasteurized [Jensen, C., S. Ethelberg, A Gervelmeyer, E. M. Mielsen, K. E. Olsen and K. Molbak (2006) "First general outbreak of Verocytotoxin-producing *Escherichia coli* O157 in Denmark" Euro. Surveill. **11**:55-58]. This report recalls a previous episode from the UK where the drinking of pasteurized milk resulted in intoxication with Shiga toxin, even though microbiological testing of the milk involved had shown that the milk had almost no live microorganisms left in it [Goh, S., C. Newman, M. Knowles, F. J. Bolton, V. Hollyoak, S. Richards, P. Daley, D. Counter, H. R. Smith and N. Keppie (2002) "E. coli O157 phage type 21/28 outbreak in North Cumbria associated with pasteurized milk," Epidemiol Infect. **209**:451-457]. Both of these papers appeared to suggest that the pasteurization process at issue was somehow defective even though no bacterial contamination of the milk was found in either case. Years later, it was discovered that Shiga toxin is sufficiently heat stable to survive the usual rigors of pasteurization [Rasooly, R. and P. M. Do (2010) "Shiga toxin Stx2 is heat-stable and not inactivated by pasteurization" International Journal of Food Microbiology **136**:290-294]. If we know that elimination of the presumed infectious agent does not eliminate the disease, then why should food HACCP plans and clinical management personnel assume that an infectious disease is the issue?

A recent paper [Sauter, K. A. D., A. R. Melton-Celsa, Kay Larkin, M. L. Troxell, A. D. O'Brien and B. E. Magun (2008) "Mouse model of hemolytic-uremic syndrome caused by endotoxin-free Shiga toxin 2 (Stx2) and protection from lethal outcome by anti-Stx2 antibody," Infection and Immunity **76**(10):4469-4478] showed that HUS is clearly the result of an intoxication with Shiga toxin as were the previously mentioned two "experiments of nature," published three years prior to the date of this review, which describes two critically-important phenomena: (i), hemolytic uremic syndrome can be induced by Shiga toxin alone (no STEC needed) and (ii), passive immunization with antitoxin given early enough in the cycle can cure the disease so many previous authors have said is incurable. The paper by Sauter *et al*. is by no means the first instance of HUS induction by Shiga toxin alone. Nevertheless, this paper and others of its kind are not merely some arcane addendum to the story of STEC "infection." They are, in fact, the tail that wagged the dog. They tell us, with convincing clarity, that HUS may be contracted without the help of any infectious agent at all. All that is necessary is the toxin, although other bacterial components (such as the lipopolysaccharide – LPS) may either increase or suppress the activity of the toxin depending upon the details of their administration.

Sauter *et al*. found that a one nanogram dose, given at day zero, day three and day six was sufficient to induce HUS in mice on day eight (and without showing signs of neurological damage). Now, a mouse only weighs about twenty grams. So, the level of

the Stx2 intoxicant was actually quite high (50 nanograms per kilogram repeated three times). It was found that a single five nanogram dose killed the mice outright, but not from HUS. Rather, the mice died from neurologic damage. The 50% lethal dose of Stx2 was found to be 3 nanograms per twenty grams or LD50 = 3 ng/20 g = 150 ng/Kg. It was also found that a single injection of anti-Stx2 antibody could cure the mice exposed to Stx2 toxemia but only if the antibody were given on day four (or earlier) of the eight days it took the experimental animals to develop HUS. The injections in this model system were intraperitoneal (*i.e.,* directly into the sac which houses the stomach and intestines). The kidneys are located within the abdominal cavity but outside of the peritoneum. Sauter *et al.* also gave intraperitoneal injections of lethal amounts of Shiga toxin and found that mice died from neurologic disorders. These results could only be possible if Shiga toxin was able to, unaided, cross the peritoneal membrane to penetrate the blood (from where it could easily cause HUS). Such results also suggested that Shiga toxin was also able to quite effectively move across the blood brain barrier to cause its lethal effects upon brain function.

The "conventional" view held by USDA/FSIS, as deduced from actions they have taken and the statements they have made is that a bloody stool followed by HUS is the result of an infectious disease caused by STEC. If one accepts that view, then one is obliged to consider all the various STEC variants are agents of infectious disease (adulterants) and seek to identify and control their living presence in meat and other foodstuffs. Holding this viewpoint is inconsistent with reports that HUS may be caused without the benefit of STEC at all. Those who hold the infectious disease viewpoint must pay attention to and explain how these observations square with the concept of STEC as an infectious agent. These reports, published in peer-reviewed literature, should be neither ignored nor dismissed.

The other viewpoint is that STEC is a relatively noninvasive enteric bacterium, which has lately been hijacked into carrying a very dangerous toxin. This toxin is able to cause the observed sequelae (including HUS) all by itself, which must mean that STEC, while certainly a carrier of this toxin, is not a disease-causing agent as such. STEC merely carries its *stx1* and/or *stx2* containing prophage payload into the colon, where it may even reproduce somewhat. However, any STEC's most salient role is as the victim of the deadly prophage it carries. When that prophage goes lytic, it not only destroys most of the population of its carriers but, in the process, generates the release of sufficient quantities of a deadly poison to the environment where the carriers once resided. The sequelae of that release are bloody stool, the result of a toxicosis of the colon, which then may (or may not) lead to a general toxemia (of the blood). Once that toxin is in the blood, it attacks the kidney merely because the cells there happen to be much more sensitive to this toxin than other organ tissues in the human body. We recognize this result as HUS. I am unaware of even one report making the claim that any STEC ever caused a septicemia (bacteria in the blood) in those it made ill. This point would seem to confirm the observation that STEC is not very invasive.

Whichever viewpoint one subscribes to, it seems clear that *E. coli* (STEC or non-STEC) should not be tolerated in meat. Of course, good, sanitary manufacturing processes

should be followed in the slaughterhouse. Regardless, meat must be Pasteurized. Radiation is not the answer. Not only would it trigger customer discontent but within current dose limitations it may only be expected to kill about 99.9% of the population present. This level of effectiveness is not adequate.

High pressure Pasteurization (HPP) can deliver the effectiveness needed (99.999% kill or greater). Some meat producers (*e.g.,* Hormel; http://www.hormelfoods.com/newsroom/brandinfo/AboutHighPressureProcessingFS.aspx) are already using this method (as mentioned in their website). This will certainly eliminate any O157 or non-O157 STEC "adulterants" from meat. However, the possibility left open by treating STEC as an infectious agent and "adulterant" is the possibility that eliminating this so-called "pathogen" through Pasteurization without testing for contamination by Shiga toxin may still leave meat unsafe to eat and consumers at risk for HUS.

The Centers for Disease Control (CDC) has already provided a method with which to measure ricin, a toxin with the same mode of action as Shiga toxin, by use of a clever synthetic substrate for N-glycosidase activity. We can adjust the analytical sensitivity of this testing scheme in several ways, most notably by adjusting the conditions of the immunomagnetic capture and concentration of this analyte. If we find it in meat, we must have a way to deal with it. Reliance upon cooking by the consumer does not seem like a good idea. Shiga toxin is a protein and enzyme we already know will survive conditions that will Pasteurize (HTST) milk. We already have experience trying to cook other protein poisons, such as prions. We know the process often ends in failure. Part of the problem is that while the polypeptide chains of an enzyme may be denatured by heat, we know that often, such denatured polypeptides may autonomously re-fold themselves so as to recreate the very enzymatic activity we were trying to destroy.

Recently, it has been shown that Shiga toxin triggers inflammation through interaction with the complement cascade. Block the complement cascade and one ameliorates HUS. It so happens that there is already a drug that does exactly this (Eculizumab – trade name Soliris) [Lapeyraque, A. L., M. Malina, V. Fremeaux-Bacchi T. Boppel, M. Kirschfink, M. Oualhua, F. Proulx, P. Niaudet and F. Schaefer (2011) "Eculizumab in severe shiga-toxin-associated HUS," New England Journal of Medicine **364**:2561-2563]. Designed by gene jockeys and cloned for relative ease of manufacture, this drug is a synthetic antibody fragment. It is currently undergoing clinical trials as a treatment for HUS. Food manufacturers, however, may want to hold off on their collective sigh of relief that a "cure" for HUS has been found and that consequently HUS is not as dangerous as it is now when there is nothing even resembling a cure. Forbes magazine has calculated that the average yearly course of this drug is likely to cost about $409,500 USD, making it the current title-holder for most expensive drug sold in the U.S.

On the other extreme (as far as cost is concerned) is manganese [Mukhopadhyay, S. and A. D. Linstedt (2012) "Manganese blocks intracellular trafficking of Shiga toxin and protects against Shiga toxicosis," Science, 20 January, pp. 332-335, DOI: 10.1126/science 1215930]. Nodules comprising up to 30% by weight of manganese are

said to litter the ocean floor from one end of this earth to the other, so the raw material is there for the taking. However, note the use in the title of the word "toxicosis" rather that "toxemia." The reader will remember that HUS is caused by Shiga toxin after it reaches the blood (toxemia). Nevertheless, if Shiga toxin toxicosis of the colon may be controlled by manganese, then perhaps the toxin may be kept from ever getting into the bloodstream to cause a toxemia (and HUS).

What is noteworthy about both the Eculizumab and manganese reports is that these presumed curative schema work against the toxin itself. This is an indication (but not proof) that the problem at hand has everything to do with the Shiga toxin and little, if anything, to do with the presumed infectious agent, STEC, regardless of its serotype. The regulatory environment has us currently set to perform handsprings to identify STEC in meat but almost nothing to address the most proximate problem – Shiga toxin. I think we would be better advised to identify (using a variant of CDC's analytic method for ricin) and then solve the actual problem: Toxemia with Stx1 and/or Stx2.

