Chemical Inactivation of Biological Agents

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I'm going to focus in 2 different areas this morning. First, an overview of the issues surrounding inactivation of biological agents, including comments about regulatory difficulties that we have in that area and then a sampling of some of the results that we have to-date from an active project as part of the National Center for Food Protection and Defense.

This slide shows some of the agents that have been called out as agents of concern. I'm going to spend some time on anthrax later in the talk. We hear talk about smallpox, plague, cholera, Ebola virus, foot and mouth disease of course, even though it's not an issue for human health, but it certainly is an economic issue. Let's take a look at what are the most resistant infective agents compared to the least resistant infective agents. I would rank them (at least today) with prions as the most resistant to vegetative bacteria and envelope viruses as least resistant.

The vegetative forms of fungi would have resistance similar to vegetative bacteria while actual spores of the fungi would fall pretty close to where bacterial spores are in terms of resistance, at least for some of the fungi. The non-enveloped viruses are agents such as Norwalk and Foot and Mouth Disease viruses, very difficult to inactive and seem to persist longer in the environment. On the other hand, the enveloped viruses, (avian influenza and human influenza viruses are like enveloped categories) are easier to inactivate and probably not as persistent in the environment.

If you take a look at one of the few example cases — we can go back, in terms of the lessons learned, to the anthrax case. We were not prepared to effectively decontaminate for example in the case of the Hart Building, it took three treatments with chlorine dioxide to achieve adequate results. In other words, we were learning along the way — The final treatment was a high level of chlorine dioxide but also high humidity to actually get the job done in that facility. Many items were destroyed as opposed to decontaminated. Personal affects like Teddy Bears in the office were bagged and taken away and treated with ethylene oxide. Ambulance chasers with cure-alls were abundant. For example, products appeared on the internet within a short time claiming that they could actually solve this problem and many of them, if you take a look at the data that they had, were comparing vegetative cells to spores.

In one example, peracetic acid was used to get a 5-log reduction. You are talking 10 times as much compound and probably 60 times as long to get spore inactivation as compared to vegetative cell inactivation. Another thing we need to consider, and I just use this as an example, is the level of contamination necessary should probably be dependent on the infective dose of the agent. In other words, for smallpox, even though it may be a poor example because it doesn't persist very long in the environment, only a few virions can cause the disease (and this would be true for something like Norwalk as well). When you decontaminate you must achieve a fairly low level to quote "be safe" and allow the building to be reoccupied.

In the case of anthrax, we perhaps do not need to get achieve zero. If you look at an infectious inhaled dose of 10,000 spores, perhaps getting to a level of 100 spores per square meter would suffice. I think that is something we need to consider. We may not have to take it all the way to zero. We have a significant regulatory hurdle in that the EPA does not allow real or implied claims for any infectious agent that is not stated on the product's label. Very few of the commercial products we have available today have been tested against the candidate biological agents. Yet, it's a violation of the law to use an EPA registered product in a manner inconsistent with labeling.

Solution strength must be according to label; applications must be on the label. In other words, the target organism must be on the label. It's a violation of federal law for a manufacturer to make real or implied claims for efficacy against organisms which are not on the label. That creates problems when we look at some of these select agents which haven't been tested.

You can't obtain them and thus we are unable to test them. I'm not sure we'd want to test them in our lab anyway; so very few products have any of these on the label. Just to give you an example of what this law means to the people who manufacture these products, there was an incident where a small company in Washington had a sporicidal product, they had some spore formers on their label but it didn't include all of them. In order to market the product for a different use, they came out with a folder that on one side had the product brochure and on the other side a paper from a peer review journal that described testing their product against certain spores. The organism mentioned in the scientific paper was not on their label and they received a half-a-million dollar fine. So that's how specific the EPA is about what we actually can do in terms of real or implied claims. Suggested surrogates for some of these are listed here. We started our work for the Center (NCFPD) using Bacillus cereus as an anthrax surrogate but we migrated then to using strains of anthrax itself. In the case of foot and mouth disease, we really don't have a good surrogate organism. The only alternative for testing with FMDV would be working with product at Plum Island to be able to do studies on foot and mouth disease virus. Norwalk virus,
while not a terrorist threat, presents a problem because it is not cul-
turable. Feline calicivirus is a suitable surrogate for Norwalk virus and
the EPA just now recognized the protocol where if your product can
pass under certain conditions against Feline calicivirus, you can actu-
ally make a Norwalk virus claim.

These are the type of tests that are required for EPA registration.
I'm going to go through this quickly but just for illustration on what has
to be done today for those of us that manufacture disinfectants, bio-
cides, and sanitizers, to actually obtain a registration. This depicts the
AOAC germicidal detergent sanitizer test. What happens is you take
99 ml of the sanitizing solution, add 1 ml of the target organism at about
10^7/ml and after a 30-s contact time, neutralize and enumerate survi-
ors. If the product achieves a kill of 5 logs in 30 s at 25 oC. The product
may be registered as sanitizer; assuming that all of this was done under
GLP (good laboratory practices). Note here that this is not exactly what
happens in the real world. This is intimate contact between the cells
and use solution, not even on a surface, no organic load present
nor soil load versus no soil load. You can see that in the best case we only see
about a 4-log reduction. Not much reduction was achieved for egg
yolk, even with 10% available chlorine, which is essentially twice the
concentration of the Clorox that you have in the home would be
about 5% available chlorine. If the temperature rose to 20 oC you get
much better activity, except in the case of egg yolk.

We see in the case of flour there is almost no effect on sodium
hypochlorite. But in egg yolk and in whole milk we saw a significant
effect. This is at 20°C – again, this is what we're looking at in the middle
of this zero to 10% (5% is straight bleach). If you evaluate sanitizers
that are used in the food industry today, they are typically used at 100
to 200 ppm – recommended concentration for use. So we’re at 10 to 100
times higher here to get inactivation of these spores. We can improve
things somewhat in some cases with temperature and in a
one-time event it may be necessary to increase exposure time and
temperature. It's not easy but doable to get a temperature in a food
plant up to 30°C, which is going to make a lot of these chemical agents
work much faster.

The disinfection test is similar except that the organisms are put on
a carrier and then they are immersed for an extended period of time. In
most cases, 10 min in the disinfecting solution. I should point out here
you will see labels including Ecolab labels that will say, “effective in the
presence of 5% serum” or a label will call out some other organic
compound. That organic load is added to the culture media and then
dried onto the surface. Thereby, it has a little more organic load than it would
normally have; however, it’s not put in the sanitizer solution. So it’s a bit
misleading to say it’s effective in 5% or 10% organic load.

A lot of factors contribute to a failure of a decontamination effort:
selection of a biocide that wasn't effective against the agent, the biocide
may be too dilute, insufficient contact time, temperature too low — we
run into that quite often, especially in Minnesota — relative humidity
too low in the case of agents such as ethylene oxide and chlorine
dioxide (that are used as gaseous sterilants). If the humidity is low,
these products don’t work very well. You have to get up to 80 to 90%
relative humidity to really get good gaseous disinfection. Presence of
organic matter — again, in that things like residual soaps, we run into
this a lot in practical situations where we get a plant that says “we seem
to be having a problem with our quaternary sanitizer”. We go back and
investigate and basically, they fail to rinse between detergent applica-
tions, which is typically anionic surfactant and then they come back
and sanitize with the quaternary compound, which is cationic and they
essentially neutralize its effect.

Coverage and wettability of the surface can be big issues. What we
face today in the case of certain infective agents, we may be looking at
decontamination of heavy soils. For example, feces, tissue, heavy
food soils. Or let's say a plant appears to be contaminated with bot-
ullinum toxin, in this case we can probably rinse the soils down the drain
because it's not an infective agent and it's going to be diluted in the
environment. If it's an infective agent; however, we can’t follow our typical
food plant cleaning where we rinse the soil, followed with a water rinse
and then use our sanitizer. That's not appropriate because we would
be dumping infectious agent into the sewage system and who knows
where it's going to go from there. We may be faced with having to
disinfect in the presence of heavy organic loads, which is far differ-
ent from what I just showed you in terms of how these products have
been registered and their intended use on clean surfaces.

The purpose of the National Center for Food Protection and De-
fense study was to investigate under practical conditions, how
we could decontaminate these heavily contaminated surfaces? An Ecolab
employee is doing his master’s degree on this. We have changed the
methodology somewhat from what I showed you earlier. We used 400
mL of anthrax spore suspension which contained several strains of
the spores. We inoculated carriers, dried it, added the sanitizer to that,
overlaid the sanitizer on these small stainless steel discs, incubated
for 10 to 30 min at different temperatures, then neutralized and enu-
merated the survivors.

We evaluated inactivation in the presence of heavy soil loads; 50%
eyg yolk emulsion, whole milk and then a flour paste. I’m going to go
briefly through a few of those results. We picked a product generally
available, like sodium hypochlorite, because we are trying to access
what we might recommend in the home. For example, what would you
use in a refrigerator? In a home you would use something that would
be readily available. Then you’ll see some examples of peracetic acid
which is generally available in food plants today. This is anthrax at 10
°C. The total available chlorine content is on the bottom. Then the soil
load versus no soil load. You can see that in the best case we only see
about a 4-log reduction. Not much reduction was achieved for egg
yolk, even with 10% available chlorine, which is essentially twice the
concentration of the Clorox that you have in the home would be
about 5% available chlorine. If the temperature rose to 20°C you get
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one-time event it may be necessary to increase exposure time and
temperature. It's not easy but doable to get a temperature in a food
plant up to 30°C, which is going to make a lot of these chemical agents
work much faster.

This data represents 30°C for ten minutes using sodium hypochlor-
rite. If you increase the time to 30 minutes, obviously you increase the
level of kill. Peracetic acid at 10 min and 10°C produces somewhat
better results. The scale on the bottom has changed from 0-to-10%
to 0-to-4% so we’re getting reasonably good activity in the 2 to 3%
range. Remember, however, that for products on the market today
that contain peracetic acid, the recommended use solution level is some-
where between 150 to 250 ppm. So again, if we were to do this today,
we would be in violation of federal law. I’m not sure who would say
anything if you were in the middle of a crisis, but that’s the situation we
face now. At 20°C, the results improve substantially. At 30°C, good
inactivation is achieved and in this case you are talking 500 ppm (or
0.5%). If you increase the temperature and time again, you get bet-
ter activity.

Here's the comparison, head-to-head, of sodium hypochlorite and
peracetic acid. After 10-minutes exposure at 20°C, the dotted lines are
sodium hypochlorite and the solid lines are peracetic acid. These results
indicate it's doable, you can change the concentration, the temperature
and the time to get the result; however we’re at far greater levels and
times than we are in terms of what we do today. If you use these
concentrations in a food plant there is a risk of damaging some of the
equipment. Maybe we should go back, even though nobody uses it in
this country anymore, to when we disinfected fresh mushroom farms
in-between crops of with formaldehyde. We may not apply it the same
way we did in those days, but for a one-time event, we should probably
go back and take a look at formaldehyde as a possible decontamina-
tion agent.

What about some of the other products that were tested? The
quaternary compound was not effective even at 7.5%. We saw those
sanitizers or disinfectants that operate by an oxidizing mechanism
perform better in these situations than did those that act by cell mod-
ification. Hydrogen peroxide actually worked fairly well.
Chemical Inactivation of Biological Agents

Bruce R. Cords
Ecolab

November 3, 2005
### Some Biological Agents of Concern

<table>
<thead>
<tr>
<th>Organism</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. anthracis</em></td>
<td>Anthrax</td>
</tr>
<tr>
<td><em>Variola major</em></td>
<td>Smallpox</td>
</tr>
<tr>
<td><em>Yersinia pestis</em></td>
<td>Plague</td>
</tr>
<tr>
<td><em>Vibrio cholera</em></td>
<td>Cholera</td>
</tr>
<tr>
<td><em>Filovirdia</em></td>
<td>Ebola</td>
</tr>
<tr>
<td><em>Aphthovirus</em></td>
<td>Foot &amp; Mouth Disease</td>
</tr>
<tr>
<td>Botulinum Toxin</td>
<td>Botulism</td>
</tr>
</tbody>
</table>
Resistance to Biocides

PRIONS

BACTERIAL SPORES

PROTOZOA CYST/OOCYSTS  NON-ENVELOPED VIRUSES

MYCOBACTERIUM

FUNGI (VEGETATIVE)

VEGETATIVE BACTERIA  ENVELOPED VIRUSES
Lessons Learned from the Anthrax Case

1. We were not prepared for decontamination (e.g. Hart Building).
2. It took three treatments with ClO₂ to achieve adequate results.
3. Many items were destroyed as opposed to decontaminated.
4. Ambulance chasers with “cure-alls” were abundant (e.g. spores vs. vegetative).
Bacillus anthracis Spores vs. Vegetative Cells

Peracetic Acid 5-log Reduction

- Spores: 2,500 ppm for 30 min.
- Vegetative: 150 ppm for 30 sec.
### Level of Decontamination Necessary Dependent on Infectious Dose

<table>
<thead>
<tr>
<th>Disease</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smallpox</td>
<td>A few virions can induce disease</td>
</tr>
<tr>
<td>Anthrax</td>
<td>Infectious dose (inhaled) may be 10,000 spores</td>
</tr>
</tbody>
</table>

- Smallpox decontamination to 100 virons/m² not acceptable
- Anthrax decontamination to 100 spores/m² probably a safe level
Regulatory Hurdle

- EPA does not allow real or implied claims for any infectious agent that is not stated on the product label.

- Very few commercial products have been tested against the candidate biological agents.
Legal Issues

1. It is a violation of Federal law to use an EPA registered product in a manner inconsistent with its labeling.
   • Solution strength must be according to label
   • Applications must be on the label

2. It is a violation of Federal law for a manufacturer to make real or implied claims for efficacy against organisms which are not on the label.
   • Creates problems when we encounter:
     • FMDV
     • Anthrax
     • Norwalk
     • SARS
     • Avian Influenza H5N1
<table>
<thead>
<tr>
<th>Agent</th>
<th>Surrogate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variola major (Smallpox)</td>
<td>Vaccinia virus</td>
</tr>
<tr>
<td><em>Yersinia pestis</em> (Plague)</td>
<td><em>Yersinia pseudotuberculosis</em></td>
</tr>
<tr>
<td><em>Bacillus anthracis</em> (Anthrax)</td>
<td>B. subtilis  B. cereus  B. globigii</td>
</tr>
<tr>
<td>Foot and Mouth Disease Virus</td>
<td>?</td>
</tr>
<tr>
<td>Norwalk Virus</td>
<td>Feline calicivirus</td>
</tr>
<tr>
<td>Product</td>
<td>Test</td>
</tr>
<tr>
<td>-----------------------</td>
<td>---------------</td>
</tr>
<tr>
<td>General disinfectant</td>
<td>AOAC Use</td>
</tr>
<tr>
<td></td>
<td>Dilution</td>
</tr>
<tr>
<td>Hospital disinfectant</td>
<td>AOAC Use</td>
</tr>
<tr>
<td></td>
<td>Dilution</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Sporicidal</td>
<td>AOAC</td>
</tr>
<tr>
<td></td>
<td>Sporicidal</td>
</tr>
</tbody>
</table>
Food Contact Surface Sanitizer

AOAC Germicidal Detergent Sanitizer Test

99 ml Sanitizer Use-Solution 25°C

Add 1 ml of *E.coli* or *S.aureus* (minimum of $7.5 \times 10^7$ CFU/ml)

30 second Contact Time

Enumerate Survivors

Neutralize 1 ml

Required Efficacy: 99.999% Kill in 30 seconds at 25°C

Efficacy: 99.999% Kill in 30 seconds at 25°C
AOAC Use Dilution Test

10 ml Sanitizer Use-Solution 20°C

Add 1 carrier with dried on *S. aureus*, *S. choleraesuis* or *P. aeruginosa*

10 minute Contact Time

Place into a neutralizer and growth medium

Incubate for 48 hours and examine for growth

Required Efficacy: No growth on 59 out of 60 carriers at 20°C
Factors Contributing to Failure of Decontamination

Disinfectant/Biocide:

- Selection of biocide not effective against infectious agent
- Biocide too dilute
- Insufficient contact time
- Temperature too low*
- Relative humidity too low
  - (gaseous disinfectants)
Factors Contributing To Failure of Decontamination

Environmental Factors:

- Presence of organic matter*
- Inactivation of QAC’s by residual soaps and detergents
- Incorrect application/coverage
- Inadequate treatment of water supply
- “Wettability” of surface
Decontamination on Farms; In Processing Plants

Because of highly infective nature of the virus, and known environmental survival, decontamination in presence of heavy soil (feces, feathers, tissues) may be required.

These conditions are far different than those under which testing for regulatory approval are conducted.
In the case of certain infective agents, decontamination in presence of heavy soil (feces, feathers, tissues) may be required.

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Study No: 910F2376745 – Ecolab

Development of Time/Temperature Concentration Matrix for Inactivation of Infectious Bioterrorism Agents by Chemical Biocides

Student: John Hilgren – Ecolab

Co-Advisors: Katherine Swanson – Ecolab
Francisco Diez-Gonzalez – University of Minnesota
Inoculate Carriers

Dry

Sanitizer

Neutralizer

Enumerate

400µl Spore Suspension + 400µl Water or Food

20 µL

100 µL

60-90 min. Ambient

10 or 30 min. at 10, 20 or 30°C

Vortex mix & pour plate

32°C / 48 hrs

400µl Spore Suspension + 400µl Water or Food

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20 µL

100 µL

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10 or 30 min. at 10, 20 or 30°C

Vortex mix & pour plate

32°C / 48 hrs

ASTM Standard Method E 2197-02 (ASTM International, West Conshohocken, PA)
## Table 2 - Test food composition

<table>
<thead>
<tr>
<th>Food</th>
<th>Protein (%)</th>
<th>Fat (%)</th>
<th>Starch (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50% Egg-yolk emulsion</td>
<td>8.8</td>
<td>16.3</td>
<td>0</td>
</tr>
<tr>
<td>Whole milk</td>
<td>3.4</td>
<td>3.5</td>
<td>0</td>
</tr>
<tr>
<td>10% Flour paste</td>
<td>1.2</td>
<td>0.1</td>
<td>7.8</td>
</tr>
</tbody>
</table>
Sodium hypochlorite – 10 minutes

Average of *B. anthracis* strains – 10°C

![Graph showing the effect of total available chlorine on the log CFU/carrier for different food types.](image-url)

- **Log CFU/carrier**
  - Egg yolk
  - Whole milk
  - Flour
  - No food

- **Total available chlorine (%)**
  - 0
  - 2
  - 4
  - 6
  - 8
  - 10
Sodium hypochlorite – 10 minutes

Average of *B. anthracis* strains – 20°C

![Graph showing the effectiveness of sodium hypochlorite on different food carriers at 20°C.](image)

- **Y-axis:** Log CFU/carrier
- **X-axis:** Total available chlorine (%)

Lines represent:
- **Black diamond:** Egg yolk
- **Yellow triangle:** Whole milk
- **Pink square:** Flour
- **Cyan circle:** No food
Sodium hypochlorite – 10 minutes

Average of *B. anthracis* strains – 30°C

**Graph:**
- **Y-axis:** Log CFU/carrier
- **X-axis:** Total available chlorine (%)
- **Data Points:**
  - Egg yolk
  - Whole milk
  - Flour
  - No food

**Legend:**
- Black diamond: Egg yolk
- Yellow triangle: Whole milk
- Pink square: Flour
- Cyan circle: No food
Sodium hypochlorite – 30 minutes

Average of *B. anthracis* strains – 30°C

- Egg yolk
- Whole milk
- Flour
- No food

Log CFU/carrier vs. Total available chlorine (%)
Peroxyacetic acid – 10 minutes

Average of *B. anthracis* strains – 10°C

- Egg yolk
- Whole milk
- Flour
- No food

Log CFU/carrier vs. Peroxyacetic acid (%)
Peroxyacetic acid – 10 minutes

Average of *B. anthracis* strains – 20°C

Graph showing the effect of peroxyacetic acid on the log CFU/carrier of *B. anthracis* strains in different carriers at 20°C. The y-axis represents the log CFU/carrier, and the x-axis represents the percentage of peroxyacetic acid. The graph includes data for egg yolk, whole milk, flour, and no food, with the relative effectiveness of each carrier shown in different colors and markers.
Peroxyacetic acid – 10 minutes

Average of *B. anthracis* strains – 30°C

![Graph showing the effectiveness of peroxyacetic acid on different food carriers at 30°C.](image)

- Egg yolk
- Whole milk
- Flour
- No food

Log CFU/canrier vs. Peroxyacetic acid (%)
Peroxyacetic acid – 30 minutes

Average of B. anthracis strains – 30°C
Comparison of sodium hypochlorite and per oxyacetic acid – 10 minutes

Average of *B. anthracis* strains – 20°C

Log CFU/carrier vs. Active ingredient (%) graph showing the effectiveness of sodium hypochlorite and per oxyacetic acid on egg yolk and flour carriers at 20°C.
Other results to date

- **Quaternary ammonium chloride**
  - Undiluted product (7.5% active) *not effective* under any conditions
  - Typically used at 0.04 to 0.08%

- **Iodophor**
  - Undiluted product (2% active) *not effective* under any conditions
  - Typically used at 0.025 to 0.01%

- **Acidified Sodium Chlorite**
  - Undiluted product (0.76% active) *not effective* when egg yolk present
  - Typically used at 0.10 to 0.15%
  - Feasible if no food/flour and $\geq 20^\circ C$ treatment for $\geq 30$ minutes
Other results to date (continued)

- **Hydrogen Peroxide**
  - Available at various concentration up to 50%, use levels vary widely
  - Foods caused little to no impact, 15 to 25% effective at 20°C

- **Peroxy/fatty acid**
  - Undiluted product contained 5% peroxyacid, typically used at 0.01 to 0.03%
  - Results similar to peroxyacetic acid but lower levels needed - except when egg yolk present
QUESTIONS