Analysis of virulence and antibiotic resistance mechanisms of *Salmonella* and development of intervention strategies

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Focus of our research program

The effects of antibiotics on *Salmonella*
- Horizontal gene transfer by bacteriophage induction
- Activation of virulence mechanisms
- Enhanced resistance due to gene duplication and amplification

Alternatives to antibiotics and *Salmonella* interventions
- *Salmonella* DIVA vaccine
- Beneficial prebiotics & probiotics
- Feed additives
Antibiotics
The 20th century wonder drug

World Health Organization (WHO) considers bacterial resistance to antibiotics a serious and growing threat
Our time with ANTIBIOTICS is running out.

Antibiotics are in danger of losing their effectiveness due to misuse and overuse, and in many cases they won’t work anymore.

NATIONAL STRATEGY FOR COMBATING ANTIBIOTIC-RESISTANT BACTERIA

Vision: The United States will work domestically and internationally to prevent, detect, and control illness and death related to infections caused by antibiotic-resistant bacteria by implementing measures to mitigate the emergence and spread of antibiotic resistance and ensuring the continued availability of therapeutics for the treatment of bacterial infections.

September 2014

ANTIBIOTIC RESISTANCE THREATS in the United States, 2013

Regulatory—FDA

- Guidance #209, #213
  - Voluntary, no growth promoters after 2016
- Veterinary Feed Directive
  - Help move OTC to Rx
Non-typhoidal Salmonella (serotypes other than Typhi, Paratyphi A, Paratyphi B, and Paratyphi C) usually causes diarrhea (sometimes bloody), fever, and abdominal cramps. Some infections spread to the blood and can have life-threatening complications.

RESISTANCE OF CONCERN
Physicians rely on drugs, such as ceftriaxone and ciprofloxacin, for treating patients with complicated Salmonella infections. Resistant infections are more severe and have higher hospitalization rates. Non-typhoidal Salmonella is showing resistance to:

- Ceftriaxone resistance
- Ciprofloxacin resistance or partial resistance
- Resistance to 5 or more antibiotic classes
- Any resistance pattern above

Annual estimations in the U.S.
1.2 million cases
23,000 hospitalizations
450 deaths
$365 million associated costs
The effects of antibiotics on Salmonella

Horizontal gene transfer by bacteriophage induction

Activation of virulence mechanisms
Horizontal gene transfer by bacteriophage induction
Carbadox in swine production

Carbadox is an antibiotic fed to 1/3 of nursery age pigs in the U.S. for the control of enteric diseases in swine at weaning, e.g., Brachyspira hyodysenteriae

Carbadox is mutagenic and has a 42 day withdrawal period prior to slaughter

It has no analog in humans; unclear if or how it will be regulated in the future
Carbadox activates bacteriophage-induced cell lysis and phage release in *Salmonella*

What is a bacteriophage (aka, prophage or phage)?
- Viruses that infect and replicate within bacteria; very common and very diverse

Many *Salmonella* contain multiple prophage
- Some are inducible (e.g. stress response)
- Some can package and transfer host DNA (generalized transduction)
Carbadox activates bacteriophage-induced cell lysis and phage release in *Salmonella* (2)
Carbadox induces phage-mediated, horizontal gene transfer of virulence genes and antibiotic resistance genes in multidrug-resistant (MDR) S. Typhimurium.

### Generalized Transduction

<table>
<thead>
<tr>
<th>Donor Strain</th>
<th>Not Induced</th>
<th>Carbadox Induced</th>
</tr>
</thead>
<tbody>
<tr>
<td>LT2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>UK1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SL1344</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>(\chi_{4232})</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DT104-NCTC13348</td>
<td>0</td>
<td>6 ± 1.7</td>
</tr>
<tr>
<td>DT104-530</td>
<td>0</td>
<td>291 ± 100.8</td>
</tr>
<tr>
<td>DT104b-5414</td>
<td>&lt;1</td>
<td>117 ± 52.8</td>
</tr>
<tr>
<td>DT120-150</td>
<td>0</td>
<td>124 ± 19.3</td>
</tr>
<tr>
<td>DT120-305</td>
<td>&lt;1</td>
<td>420 ± 94.8</td>
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<tr>
<td>DT120-613</td>
<td>&lt;1</td>
<td>86 ± 16.7</td>
</tr>
<tr>
<td>DT193-1434</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DT208-2348</td>
<td>0</td>
<td>0</td>
</tr>
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</table>

Average frequency of generalized transduction per 0.5 ml of lysate from numerous S. Typhimurium donor strains into BBS 243.
Do other *Salmonella* serovars contain generalized transducing phage capable of horizontal gene transfer?

DNA sequence analysis suggests yes:


Based on genome sequence scanning by Sherwood Casjens and/or genome alignments to Prophage 1 *int* in DT104 using BioCyc*
Can other antibiotics induce phage-mediated gene transfer from MDR *Salmonella*?

Fluoroquinolones - broad spectrum antibiotic

Human Medicine
- Ciprofloxacin

Veterinary respiratory diseases
- Enrofloxacin (Baytril®)
- Danofloxacin (Advocin™)

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**Figure:**

![Graph showing transductants/ml lysate for different isolates and antibiotics.]

- **Isolate**:
  - L12
  - DT120
  - DT104

- **Y-axis**: Transductants/ml lysate
- **X-axis**: Isolate

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Control</th>
<th>Ciprofloxacin</th>
<th>Enrofloxacin</th>
<th>Danofloxacin</th>
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<td>DT104</td>
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Short Communication

Fluoroquinolone induction of phage-mediated gene transfer in multidrug-resistant *Salmonella*

Bradley L. Bearson a, b, Brian W. Brunelle b
The Take-Home Message

Certain *Salmonella* strains including MDR *S. Typhimurium* contain generalized transducing prophage capable of packaging and transducing *Salmonella* DNA.

Transfer of antibiotic resistance, virulence, or other fitness genes.
Antibiotic induction of *Salmonella* virulence mechanisms
National Antimicrobial Resistance Monitoring System (NARMS):
12-year average of *Salmonella* antibiotic resistance in humans and animal commodities
Virulence gene regulation *in vitro*

What are the exposure effects of tetracycline/chlortetracycline on MDR *Salmonella*?

**Invasion**

In early log-phase growth when *Salmonella* is typically non-invasive, tetracycline induces invasion gene expression and accelerates cellular invasion in some MDR *Salmonella*.

Induction of invasion gene expression (37 genes)

<table>
<thead>
<tr>
<th>Gene</th>
<th>0 μg/ml</th>
<th>1 μg/ml</th>
<th>4 μg/ml</th>
<th>16 μg/ml</th>
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<tbody>
<tr>
<td><em>avrA</em></td>
<td>6.0</td>
<td>8.2</td>
<td>1.2</td>
<td>2.6</td>
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<tr>
<td><em>hilA</em></td>
<td>13.4</td>
<td>14.8</td>
<td>1.4</td>
<td>4.0</td>
</tr>
<tr>
<td><em>hilC</em></td>
<td>-1.5</td>
<td>1.1</td>
<td>-8.5</td>
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</tr>
<tr>
<td><em>hilD</em></td>
<td>6.1</td>
<td>6.6</td>
<td>1.2</td>
<td>3.4</td>
</tr>
<tr>
<td><em>iagB</em></td>
<td>20.2</td>
<td>17.0</td>
<td>3.3</td>
<td>6.4</td>
</tr>
<tr>
<td><em>invA</em></td>
<td>9.4</td>
<td>11.2</td>
<td>2.7</td>
<td>3.5</td>
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<td>5.9</td>
<td>-1.0</td>
<td>3.2</td>
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<td>3.2</td>
<td>3.1</td>
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<td>1.3</td>
<td>1.7</td>
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<td><em>invF</em></td>
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<td>1.7</td>
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<td>12.3</td>
<td>1.1</td>
<td>1.4</td>
</tr>
<tr>
<td><em>invI</em></td>
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<td>4.3</td>
<td>-1.3</td>
<td>2.6</td>
</tr>
<tr>
<td><em>invJ</em></td>
<td>18.1</td>
<td>11.0</td>
<td>2.7</td>
<td>4.9</td>
</tr>
<tr>
<td><em>orgAa</em></td>
<td>12.1</td>
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</tr>
<tr>
<td><em>orgAb</em></td>
<td>10.4</td>
<td>10.2</td>
<td>3.5</td>
<td>4.6</td>
</tr>
<tr>
<td><em>prgH</em></td>
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<td>1.9</td>
<td>1.5</td>
<td>1.4</td>
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<td><em>prgl</em></td>
<td>5.9</td>
<td>6.5</td>
<td>1.5</td>
<td>2.6</td>
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<tr>
<td><em>prgJ</em></td>
<td>3.0</td>
<td>2.0</td>
<td>-2.2</td>
<td>-1.2</td>
</tr>
</tbody>
</table>

* Early log invasion of Hep-2 cells

What are the effects of tetracycline exposure on MDR Salmonella?

**Attachment**

Fimbriae are appendages involved in attachment 13 fimbrial operons in Salmonella Typhimurium

Only 3 are considered to be inducible in vitro
- csg, fim, and pef

6 are associated with persistence in mice
- bcf, lpf, stb, stc, std, and sth

Tetracycline induced 10 operons
- bcf, csg, fim, lpf, pef, saf, stb, stc, std, and sth

Electron Microscopy: no Tetracycline

1434, no tetracycline
Electron Microscopy: with Tetracycline

1434 16 ug/ml tetracycline
The Take-Home Message

Effects of tetracycline/chlortetracycline exposure on MDR Salmonella:

• Differentially regulated ~3,000 genes

• Accelerated progression of pathways that normally take 8+ hours, including induced invasion and attachment during early-log growth

• Activated ten fimbriae operons

Tetracycline-inducible invasion phenotype:
Provides MDR Salmonella with an additional competitive advantage that influences colonization potential and may be associated with increased morbidity among patients with MDR Salmonella infections
Our goal of investigating the effects of antibiotics on MDR Salmonella is to identify and characterize antibiotics that stimulate bacterial virulence mechanisms (as well as those that do not).

This information will assist veterinarians and food animal producers when determining the proper antibiotic therapy for infectious disease treatment that does not unintentionally complicate an illness and compromise animal management.
Alternatives to Antibiotics and *Salmonella* interventions

*Salmonella* DIVA vaccine

Beneficial prebiotics & probiotics

Feed additives
Identify on-farm intervention strategies to reduce the **human** foodborne pathogen *Salmonella* in food animals

Livestock and poultry colonized with *Salmonella* are often **asymptomatic carriers** of the **human** pathogen.

*Salmonella*-carrier animals can enter the food chain, contaminating slaughter plants and meat products.

These animals have the potential to shed the pathogen in their feces, resulting in unrecognized disease transmission/spread and contamination of the environment.
Salmonella DIVA vaccine
The *Salmonella* challenge

> 2,500 serovars

Ubiquitous in nature

Wide host range

Gastrointestinal to Systemic disease

Livestock are often asymptomatic carriers
Limitations of current *Salmonella* vaccines

**Serovar-specific protection**

Vaccination may interfere with *Salmonella* surveillance programs to identify *Salmonella*-positive herds or flocks
Our vaccine goal:
Attenuated S. Typhimurium strain
Reduction in disease and colonization
Cross-protection against multiple serovars
DIVA (Differentiation of Infected from Vaccinated Animals)

Reduce the variable, immunodominant Salmonella antigens
Increase conserved, Salmonella outer membrane proteins
Refocus the immune response by reducing LPS and flagella
Reducing serovar specific immunity: part 1
Decrease variable, immunodominant antigens

**RfaH antiterminator**

RfaH prevents premature transcription termination of long mRNA operons, including immunodominant:

- LPS (lipopolysaccharide)
- Flagellar/chemotaxis

Differentiate the >2,500 *Salmonella* serovars by their differences in LPS and flagella

*Kauffmann and White classification scheme*
Reducing serovar specific immunity: part 2

Increase conserved outer membrane proteins

Small Regulatory RNA (sRNA)

- 50–250 nucleotides in length
- Generally untranslated RNA
- Encoded in intergenic regions
- Regulate their targets at post-transcriptional level

Salmonella Typhimurium BBS 866 live vaccine in swine

**Attenuated** in pigs

**Differentiation** of infected from vaccinated animals (DIVA)

**Cross-protective** against wild-type challenge Choleraesuis (systemic disease) and Typhimurium (gastrointestinal disease)

- Reduced clinical disease (body temperature and diarrhea)
- Reduced fecal shedding (2-log reduction in ST)
- Reduced tissue colonization (1-2 log reduction in ST & SC)
- Reduced bacteremia (blood culture, spleen, liver in SC)
- Increased average daily gain (SC)

Vaccine. 2016. 34:1241-6.
Vaccination significantly decreased *Salmonella* Heidelberg isolation from the spleen and ceca in turkeys.

- 2 days old: Vaccinate (10⁹)
- 2 weeks old: Booster vaccinate (10⁹)
- 5 weeks old: MDR Heidelberg challenge (10⁹)

### Ceca-associated S.H. at 7 Days Post-challenge

<table>
<thead>
<tr>
<th>CFU/g Tissue (Log10)</th>
<th>Mock</th>
<th>BBS 866</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
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<td></td>
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<tr>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Spleen-associated S.H. at 7 Days Post-challenge

<table>
<thead>
<tr>
<th>CFU/g Tissue (Log10)</th>
<th>Mock</th>
<th>BBS 866</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
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<tr>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
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</tr>
</tbody>
</table>

*Note: *a indicates a significant difference.
**The Take-Home Message (2)**

*Salmonella* Typhimurium BBS 866 live vaccine

**Attenuated** in pigs & turkeys

**Differentiation** of infected from vaccinated animals (DIVA)

**Cross-protective** against wild-type challenge *Cholerasuis* (pigs) and *Heidelberg* (turkeys)

Vaccine was exclusively licensed by Huvepharma in August 2017
U.S. patent was issued for the vaccine in January 2018
Analyzing the porcine intestinal microbiota in response to *Salmonella* in search for potential swine-specific probiotics
Low versus High *Salmonella* shedding in pigs

On the farm, as well as within experimental infections, the shedding of *Salmonella* from infected pigs varies from low-shedding to persistently high-shedding.

Are there differences between the microbiota composition in the gastrointestinal tract of “will be” Low-shedders and “will be” High-shedders prior to inoculation with *Salmonella* that could influence *Salmonella* colonization and persistence?

What microbiota alterations occur after introducing *Salmonella* into the GI tract and do the microbiota changes correlate with *Salmonella* shedding status?
Salmonella shedding in swine

Salmonella enterica serovar Typhimurium (1 billion)

Litter-mate, mock-inoculated controls

Day 7

<table>
<thead>
<tr>
<th>ANOSIM</th>
<th>p-value</th>
<th>R-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NI vs. LS</td>
<td>0.55</td>
<td>-0.04</td>
</tr>
<tr>
<td>NI vs. HS</td>
<td><strong>0.01</strong></td>
<td>0.37</td>
</tr>
<tr>
<td>LS vs. HS</td>
<td><strong>0.01</strong></td>
<td>0.41</td>
</tr>
</tbody>
</table>

Legend:
- **Blue** Non-inoculated
- **Green** Low Shedders
- **Red** High Shedders
Phylotype analysis

Prior to *Salmonella* challenge, the “will-be” low shedder pigs had a significantly greater relative abundance of *Ruminococcaceae*, members of which are:
- enriched in the mucosal environment
- have shown decreased abundance in other animals with gut disturbance
- produce short-chain fatty acids, including butyrate

Butyrate-producing bacteria

The diversity of butyrate-producing bacteria is different in swine that become high shedders versus low shedders.
Identify feed additives that enhance short chain fatty production in the intestinal tract to reduce *Salmonella* in swine.
Potential mechanism for reducing *Salmonella* colonization or shedding

Encouraging short change fatty acid (SCFA) production, particularly butyrate, might reduce the niche for microbes that use respiration by limiting electron acceptor availability.

Oxidation of butyrate in epithelial cells consumes most available oxygen (i.e. less available $O_2$).

SCFAs encourage robust mucus barrier:
- More AMPs, more mucus, more IgA
- Limits the chance microbes will contact host tissues and elicit inflammatory response
- Less immune derived reactive oxygen species (ROS)
Feed trial with *Salmonella* challenge

6 diets
Control
Raw Potato Starch (5%)
Acid blend: 0.3% butyric acid-propionate-medium chain fatty acid mix
ZnO (2,000 mg/kg) + CuCl (200 mg/kg)
High amylose corn starch (5%)
Beta-glucan (0.05%, yeast based)

- Ten pigs/treatment group (started feed trial at 3-weeks of age)
- After 4 weeks on the various diets, pigs were challenged with $1 \times 10^8$ CFU *Salmonella enterica* serovar 14,[5],12:i:- (feed additives continued)
- Fecal shedding was monitored for 21 days
- At necropsy, butyrate concentrations was measured in the cecal contents
Feed trial with *Salmonella* challenge (2)

Butyrate production is increased in the cecal contents of *Salmonella* challenged pigs fed raw potato starch and β-glucan.

Cumulative *Salmonella* fecal shedding over the 3-week study was significantly reduced in pigs fed raw potato starch and β-glucan.

Wilcoxon: control vs RPS, p=0.004; control vs Bglu, p=0.036

Wilcoxon: control vs RPS, p=0.013; control vs Bglu, p=0.043
Correlation between butyrate concentration in cecal contents and cumulative *Salmonella* fecal shedding

Spearman: -0.4, p=0.003
The Take-Home Message

The microbial composition of the intestinal tract can influence *Salmonella* shedding in pigs.

Feed components that promote SCFA-producing microbes (e.g. butyrate-producers) may benefit the host by enhancing swine gut health and reducing *Salmonella* colonization and shedding.

Probiotics + Prebiotic =
Thank you!

Heather Allen
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Chris Tuggle