Identification and Quantification of 6 Illegal Antibiotics in Chinese Chicken Jerky Dog Treats

New York State Department of Agriculture & Markets
Food Laboratory
Robert Sheridan
Dogs begin experiencing symptoms

- In 2007 several cases of Acquired Fanconi Syndrome in dogs were suspected to be associated with the consumption of chicken jerky treats imported from China.

- Fanconi Syndrome – kidney malfunction and can lead to death.

- Symptoms include:
  - Weight loss
  - Reduced appetite
  - Excessive thirst
  - Lethargy
  - Vomiting
  - death
Causes of Acquired Fanconi Syndrome

- Exposure to heavy metals (Pb, Hg, Cd, U..)
- Certain drugs such as cidofovir, tenofovir, outdated tetracycline
- Paraquat, Diquat
- Lysol
- Certain organic solvents such as toluene
- Lysine
- Maleic acid
- Other chemical agents
Causes of Acquired Fanconi Syndrome

- Exposure to heavy metals, inorganics (Pb, Hg, Cd, U..)
  - Microwave digestion followed by analysis by ICP–MS
  - Relatively easy to determine if they are present because the periodic table is limited.
Causes of Acquired Fanconi Syndrome

- Exposure to heavy metals (Pb, Hg, Cd, U,..)
- Certain drugs such as cidofovir, tenofovir, outdated tetracycline
- Paraquat, Diquat
- Lysol
- Certain organic solvents such as toluene
- Lysine
- Maleic acid
- Other chemical agents (organic)

About 9 million organic compounds are known to exist.
Detection of organic compounds

- Gas or Liquid chromatography provides separation of analyte of interest from co-extracted matrix interference and from other analytes

- Tandem mass spectrometry provides unambiguous detection and quantitation
Detection of organic compounds

- **Targeted screen**
  - Analytes are determined
  - Analytical conditions are determined using standards (retention time, parent mass > product mass, ion ratio...)
  - Samples are run to determine presence and quantity of analytes

- **Unknown screen**
  - Analytes are detected using means other than comparison with a standard
    - Spectral examination of suspected analyte (GC–EI spectrum searching)
    - Exact mass determination – LC–HRMS
Sample Preparation

- Bags of suspect chicken jerky are received
- Given sample number
- Typically several pieces from a bag is ground together and considered to be one sample

Extraction with organic solvent
Sample Preparation

- We decided to grind each piece separately and give each piece a unique sample number.
Why analyze treats individually?

- Prevent possible dilution of an unknown contaminant if “hot spots” exist. This makes detection of contaminants easier.

- Possibly allow us to observe differences between treats from the same bag.

- Many times one treat is the recommended serving size
Chicken jerky label

Daily maximum number of piece(s) based on your dog's weight

- Small Dogs (5-10)
- Small - Medium Dogs (11-25)
- Medium Dogs (26-50)
- Large Dogs (over 50)

* Senior Dogs have different feeding needs based on weight and age. Ask your veterinarian what's best for your dog.

Make sure your dog gets plenty of fresh water daily. In addition to a nutritionally balanced diet, daily exercise is important too.
**Chicken jerky label**

### Feeding Instructions
Feed as a snack.
Recommended feeding instructions based on dog’s weight:
Fresh drinking water should always be available.

<table>
<thead>
<tr>
<th>Dog Size</th>
<th># Pieces</th>
</tr>
</thead>
<tbody>
<tr>
<td>Under 5 lbs</td>
<td>½ - 1</td>
</tr>
<tr>
<td>5 - 10 lbs</td>
<td>1 - 2</td>
</tr>
<tr>
<td>10 - 25 lbs</td>
<td>2 - 3</td>
</tr>
<tr>
<td>25 - 50 lbs</td>
<td>3 - 4</td>
</tr>
<tr>
<td>50 - 75 lbs</td>
<td>4 - 5</td>
</tr>
<tr>
<td>Over 75 lbs</td>
<td>5 - 8</td>
</tr>
</tbody>
</table>

**INGREDIENTS:** Chicken Breast, Glycerin, Sugar, Salt, Natural Flavors, Mixed Tocopherols (a Preservative and Natural Source of Vitamin E)

The makers of Milo’s Kitchen™ dog treats do not use any artificial colors; color change in this product is due to the natural color of the ingredients.

Comments or Questions?
How likely are we to find the unknown compound?

- The compound must be
  - Extracted
  - Chromatographed
  - Ionized
  - Within mass range

<table>
<thead>
<tr>
<th>extracted</th>
<th>chromatographed</th>
<th>ionized</th>
<th>within mass range</th>
</tr>
</thead>
<tbody>
<tr>
<td>High $K_{ow}$</td>
<td>Well resolved peak</td>
<td>ESI</td>
<td>(50 - 2000 AMU)</td>
</tr>
<tr>
<td>Low $K_{ow}$</td>
<td>Unretained on column</td>
<td>Not ionized (PAH)</td>
<td>Detection</td>
</tr>
<tr>
<td>(water soluble)</td>
<td>Improper mobile phase</td>
<td></td>
<td>65 kDa</td>
</tr>
<tr>
<td>or</td>
<td>Not eluted from column</td>
<td></td>
<td>Anthrax toxin – 85 kDa</td>
</tr>
<tr>
<td>too volatile</td>
<td></td>
<td></td>
<td>Shiga toxin – 32 kDa</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Digestion</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Multiple charged</td>
</tr>
</tbody>
</table>

- Ricin - 65 kDa
- Anthrax toxin – 85 kDa
- Shiga toxin – 32 kDa
Targeted screens

- Toxin screen – 36 known toxins
  - Acetonitrile extraction
  - Analysis by LC/MS/MS

- Pesticide screen – 200 targeted pesticides
  - Acetonitrile extraction – solid phase clean up
  - Analysis by LC/MS/MS and GC/MS/MS

- Rodenticide screen – 10 targeted rodenticides
  - Acetonitrile extraction
  - Analysis by LC/MS/MS

- Mycotoxin screen – 9 mycotoxins
  - Elisa analysis

- Antibiotics screen – 38 legal and illegal veterinary drugs
  - 16 sulfonamides, 22 others
  - Acetonitrile extraction
  - UPLC/MS/MS analysis
Targeted screens

Results

- Sulfaquinoxaline 100 ppb
- Sulfamethoxazole no tolerance
- Enrofloxacin no tolerance
- Tilmicosin no tolerance
- Trimethoprim no tolerance

FDA 21CFR
Unknown peak found in chicken jerky same transitions as
Sulfachloropyridazine

285 > 156
285 > 91.8

Unknown

RT  4.09
Ion ratio  55%

Sulfachloropyridazine

RT  2.59
Ion ratio  71%
Extract containing unknown peak was sent to Keith Goodman at AB Sciex (Framingham MA) for high resolution analysis using 5600 LC/QTOF

Empirical formula determined to be

$C_{10}H_9N_4O_2SCl$ (same as sulfachloropyridazine)
Isomers of sulfachloropyridazine

4-amino-N-(5-chloropyrimidin-2-yl) benzenesulfonamide
Metanilamide,N 1–5(chloro–2–pyrimidinyl)
3-amino-N-(5-chloro–2–pyrimidinyl) benzenesulfonamide
4-amino-N-(6-chloro–3–pyrimidinyl) benzenesulfonamide
Sulfaclozine

antibiotic used in poultry production (not allowed in US)
Unknown peak found in chicken jerky
same transitions as Sulfachloropyridazine

RT 4.09
Ion ratio 55%

Unknown

Sulfaclozine
Alibaba.com = Amazon.com for industrial chemicals
25% tilmicosin oral solution for poultry
Min. Order: 3000 Barrels
FOB Price: US $2.30 / Barrel
Supply Ability: 10000 Barrel/Barrels per Day

poultry medicine / tilmicosin premix
Min. Order: 2000 Kilograms
FOB Price: US $2.4 / Day
Supply Ability: 500 Kilogram/Kilograms per Day

Tilmicosin Solution veterinary medicine chemicals
Min. Order: 5000 Boxes
Supply Ability: 1000 Box/Boxes per Day
<table>
<thead>
<tr>
<th>Product Description</th>
<th>Contact Supplier</th>
<th>Country</th>
<th>Employees</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chicken medicine/poultry medicine/Multivitamin poultry powder</td>
<td>Offline</td>
<td>China (Mainland)</td>
<td>301 - 500</td>
</tr>
<tr>
<td>Hi-effective and Veterinary and poultry medicines with Veterinary products with Vitamin AD3 E complex oral solution/Poultry drugs</td>
<td>Offline</td>
<td>China (Mainland)</td>
<td>201 - 300</td>
</tr>
<tr>
<td>Poultry medicine for prevention of avian flu</td>
<td>Offline</td>
<td>China (Mainland)</td>
<td>11 - 50</td>
</tr>
<tr>
<td>Poultry medicine, (Enrofloxacin Soluble Powder)</td>
<td>Offline</td>
<td>China (Mainland)</td>
<td>10 - 200</td>
</tr>
</tbody>
</table>

**Note:** Details such as Min. Order, FOB Price, and Supply ability vary across products.

**Contact Details:**
- Hobel New Century Pharmaceutical Co., Ltd.
- Weifang Premier Animal Pharmaceutical Industries Co., Ltd.
- Sichuan Jiao Kang Plastic Technology Co., Ltd.
- Shenyang Tiangeng Animal Husbandry Research Factory
<table>
<thead>
<tr>
<th>Subsample</th>
<th>Analyte (ppb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>sulfaclozine 3.47</td>
</tr>
<tr>
<td></td>
<td>sulfaquinoxaline 7.54</td>
</tr>
<tr>
<td></td>
<td>enrofloxacin &lt;3</td>
</tr>
<tr>
<td>2</td>
<td>sulfaclozine 751</td>
</tr>
<tr>
<td></td>
<td>sulfaquinoxaline 828</td>
</tr>
<tr>
<td></td>
<td>enrofloxacin &lt;3</td>
</tr>
<tr>
<td>3</td>
<td>sulfaclozine 728</td>
</tr>
<tr>
<td></td>
<td>sulfaquinoxaline 828</td>
</tr>
<tr>
<td></td>
<td>enrofloxacin &lt;3</td>
</tr>
<tr>
<td>4</td>
<td>sulfaclozine 12.0</td>
</tr>
<tr>
<td></td>
<td>sulfaquinoxaline 12.1</td>
</tr>
<tr>
<td></td>
<td>Tilmicosin &lt;3</td>
</tr>
<tr>
<td></td>
<td>enrofloxacin &lt;3</td>
</tr>
</tbody>
</table>
# Highest concentrations found

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Concentration</th>
<th>FDA Tolerance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfaclozine</td>
<td>2000 ppb</td>
<td>0</td>
</tr>
<tr>
<td>Sulfaquinoxaline</td>
<td>828 ppb</td>
<td>100 ppb</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>132 ppb</td>
<td>0</td>
</tr>
<tr>
<td>Sulfamethoxazole</td>
<td>5.2 ppb</td>
<td>0</td>
</tr>
<tr>
<td>Tilmicosin</td>
<td>528 ppb</td>
<td>0</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>41 ppb</td>
<td>0</td>
</tr>
</tbody>
</table>
All major brands voluntarily removed from sale throughout US

- No indication the illegal antibiotics were responsible for dog illnesses
- Consistently above tolerance
- Antibiotic misuse could contribute to pathogenic bacteria resistance
Fluoroquinolone-Resistant *Campylobacter* Species and the Withdrawal of Fluoroquinolones from Use in Poultry: A Public Health Success Story

Jennifer M. Nelson, Tom M. Chiller, John H. Powers, and Frederick J. Angulo

1Enteric Diseases Epidemiology Branch, Division of Foodborne, Bacterial and Mycotic Diseases, National Center for Zoonotic, Vectorborne, and Enteric Diseases, Centers for Disease Control and Prevention, and Atlanta Research and Education Foundation, Atlanta, Georgia; and 2National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland

*Campylobacter* species cause 1.4 million infections each year in the United States. Fluoroquinolones (e.g., ciprofloxacin) are commonly used in adults with *Campylobacter* infection and other infections. Fluoroquinolones (e.g., enrofloxacin) are also used in veterinary medicine.

Human infections with fluoroquinolone-resistant *Campylobacter* species have become increasingly common and are associated with consumption of poultry. These findings, along with other data, prompted the US Food and Drug Administration to propose the withdrawal of fluoroquinolone use in poultry in 2000. A lengthy legal hearing concluded with an order to withdraw enrofloxacin from use in poultry (effective in September 2005). Clinicians are likely to continue to encounter patients with fluoroquinolone-resistant *Campylobacter* infection and other enteric infection because of the continued circulation of fluoroquinolone-resistant *Campylobacter* species in poultry flocks and in persons returning from foreign travel who have acquired a fluoroquinolone-resistant enteric infection while abroad. Judicious use of fluoroquinolones and other antimicrobial agents in human and veterinary medicine is essential to preserve the efficacy of these important chemotherapeutic agents.
African and EU See Rising Level of Antibiotic-Resistant Salmonella

By James Andrews | June 20, 2013

Strains of one increasingly antibiotic-resistant Salmonella serotype have seen a “rapid worldwide spread,” according to a study published by researchers at the Institut Pasteur in Paris and Morocco.

Antibiotic-resistant Salmonella Kentucky, first isolated in 2002 in a French tourist who had visited Egypt, has now “spread at an astonishing rate throughout Africa and the Middle East in the space of only a few years,” the study’s authors claim.

The bacterium has also already been found in farmed-raised turkeys in Europe, though it is not clear based on available information if those turkeys were imported or grown domestically. In a summary of the study, the lead author said he worries that the resistant strain may soon spread to European poultry farms.

This study comes on the heels of a report out of Canada calling antibiotic-resistant Salmonella Kentucky a rare but “growing concern” in Canadian health. That study found that between 2003 and 2009, 30 percent of Salmonella Kentucky isolates from Canadian patients were resistant to the antibiotic ciprofloxacin.

Those Canadian infections, however, were not associated with any retail food sold in Canada. Instead, every patient with available travel information had visited an African country within a week of developing symptoms.

According to the authors of the Pasteur study, the resistant bacterium has continued to spread through Mediterranean countries, particularly Morocco, infecting hundreds of patients each year.

“In addition, the authors of this study made the troubling observation that a number of strains recently acquired in the Mediterranean Basin are showing a range of resistance towards all antibiotic classes used to treat severe cases of salmonellosis,” the study’s summary read.

The main vehicle of transmission for antibiotic-resistant Salmonella Kentucky from African and Middle Eastern countries appears to be chickens and turkeys. The authors said the resistance is believed to be caused by “the massive overuse” of antibiotics in African poultry farming.

According to a May 2013 report by the Center for Science in the Public Interest, the U.S. saw 55 outbreaks of antibiotic-resistant pathogens between 1973 and 2011. Contaminated dairy products and ground beef accounted for the majority of those outbreaks. Antibiotic-resistant Salmonella strains accounted for 50 (91 percent) of those drug-resistant outbreaks, though none of them were Salmonella Kentucky. At least 35 (64 percent) of those were resistant to five or more antibiotics.

On Monday, Congresswoman Louise Slaughter (D-NY), the only microbiologist in Congress, wrote a letter to President Obama urging him to “pay special attention to issues of antibiotic resistance” at this week’s G-8 Summit in Northern Ireland. Slaughter also suggested the President consider stronger limits on antibiotic use in animal agriculture.

David Willetts, Britain’s science minister, is expected to use his platform at the G-8 meeting to propose new measures to curb the overuse of antibiotics by both healthcare professionals and farmers alike.

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Recent findings

- Recent acquisition of Sciex 5600 triple TOF
- Allows for identification of compounds without comparison to a standard.
  - Exact mass determination
  - High resolution product ion library searching
  - Empirical formula finding
Standards are purchased
LC/MS/MS analysis method is developed
Extraction method is optimized
Samples are extracted with method
Samples are analyzed along with standards
Analytes are confirmed with tandem MS
  ◦ RT
  ◦ Ion ratio comparison
Analytes are quantified
<table>
<thead>
<tr>
<th></th>
<th>Amantadine</th>
<th>DEET</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detection frequency</td>
<td>36%</td>
<td>38%</td>
</tr>
<tr>
<td>Highest concentration</td>
<td>882 ng/g</td>
<td>572 ng/g</td>
</tr>
</tbody>
</table>
Amantadine
- Antiviral drug only approved for human use
- Chinese poultry farmers were suspected of misuse in 2005 for prevention of avian flu
- H5N1 strains in China are now resistant

DEET
- Insect repellent/ pesticide
- Acetylcholinesterase inhibitor in insects and mammals
No connection has been made between any of the 8 compounds detected and the illnesses.

Many of the detections represent misuse.

Investigation continues.
Special thanks to:

- Kristen Hafler
- Jennifer Mirabile
- Kendal Harr
Questions ?