Laboratory Simulation of *Salmonella* Contamination and Recovery on Bermudagrass

M.R. McLaughlin and J.P. Brooks

USDA, ARS, Waste Management and Forage Research Unit, Mississippi State, MS

Abstract: Confined swine feeding operations in the southeastern US flush manure into lagoons and the effluent is applied to forage crops cut for hay. Bermudagrass, *Cynodon dactylon* (L.) Pers., is the most widely used of these summer forages. Application is by irrigation using overhead nozzle center pivot systems or traveling gun reel systems. Although swine manure is known to contain *Salmonella*, little is known of the incidence and significance of *Salmonella* in effluents applied to bermudagrass. A laboratory method was developed to test the application and recovery of *Salmonella* on bermudagrass preliminary to examining this problem in the field. EPA (U.S. Environmental Protection Agency) "worst case water" (WCW) was used to simulate lagoon effluent. WCW with high turbidity and organic matter was originally developed to test water filtration devices. In the present application, WCW was loaded with known levels of *Salmonella*. *Salmonella* grown in trypticase soy broth was concentrated and washed by three cycles of centrifugation (5K x g, 15 min, 10C) and suspension in phosphate-buffered saline (PBS). Cell suspensions were counted (colony forming units, cfu/ml) by dilution plating on trypticase soy agar (TSA) and stored at 4C. To simulate irrigation, freshly cut leaves were trimmed to 6 cm in length and 3 cm of the leaf tips dipped individually 10X in *Salmonella*-loaded WCW. Treated leaves were held on sterile filter paper wetted with sterile distilled water inside glass Petri dishes at room temperature. Individual leaves, removed at intervals for *Salmonella* testing, were placed tip down in sterile 5-ml plastic culture tubes containing 1 ml of sterile PBS. *Salmonella* were eluted by vigorous swirling in tightly capped tubes using a vortex mixer (15 sec, 2X) and counted on TSA spread plates. Recovery from leaves exposed to 10^6 cfu/ml, varied widely (0 - 10^4 cfu/leaf) within and among cultivars.

Salmonella levels in swine lagoon effluent*

Lagoon	Estimated Salmonella Level MPN/100ml	SD
1	142	9
2	269	5
3	621	3
4	76	3
5	82	2
6	75	2

*1.0, 0.1, and 0.01 ml (3 tubes each) of undiluted effluent was added to 10 ml buffered peptone water and incubated for 24 hrs at 35C in a water bath, then 0.5 ml of each tube was transferred to 10 ml of Rappaport-Vassiliadis R10 broth and incubated at 42C +/- 1C for 24 to 36 hrs in a water bath. Positive tubes were transferred to modified semisolid Rappaport-Vassiliadis (0.1 ml in 3 drops each to 6-well cell culture plates) and incubated at 42C in a "dry" incubator. All RVR10 tubes (positive or negative) were transferred to a 96-well PCR plate (0.1 ml of each tube) and heated to 98C for 10 minutes in a thermal cycler. A 10:1or 10:2 dilution was made and 2 µl was subjected to real time PCR with *spaQ* primers. Most probable numbers (MPN) of *Salmonella* colony forming units per 100 ml effluent sample were determined from real time PCR results and the data combined (6 samples per lagoon) to estimate *Salmonella* levels (log average or geometric mean MPN/100 ml and std dev) for each lagoon.

*Salmonella** Isolates

Ref No.	ATCC No.	Serovar	Antigenic Formula
R05	13076	Enteritidis	1,9,12:[f],g,m,[p]:[1,7]
R21	43971	Typhimurium	1,4,[5],12:i:1,2
R42	BAA-707	Agona	1,4,[5],12:f,g,s:[1,2]
R44	BAA-709	Michigan	17:1,v:1,5
R46	BAA-711	Gaminara	16:d:1,7

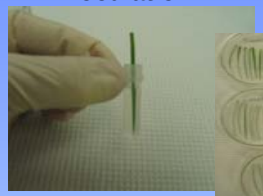
**Salmonella enterica* subsp. *enterica (ex Kauffman and Edwards) Le Minor and Popoff*

EPA Worst Case Water (WCW)

Total Dissolved Solids (TDS = 1500 mg/L)
Sea Salts (Sigma S9883)……..1.5 g/L
Total Organic Carbon (TOC = 10 mg/ml)
Humic Acid (Sigma-Aldrich H16752)..10 mg/L
Turbidity (30 NTU)
Fine Test Dust (ISO 12103-1)
(Powder Technology Inc.)…… 30 NTU

References: Gerba & Naranjo. Wilderness & Environmental Medicine 11:12-16. (2000).
USEPA. Guide Standard & Protocol for Testing Microbiological Water Purifiers. (1987).

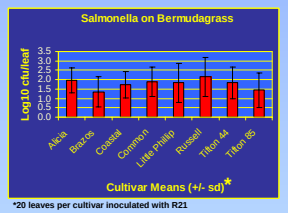
Inoculation



Incubation

Elution

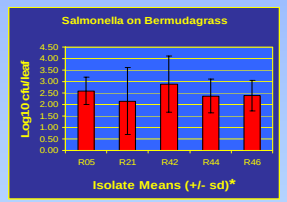
Enumeration



*20 leaves per cultivar inoculated with R21

ANOVA

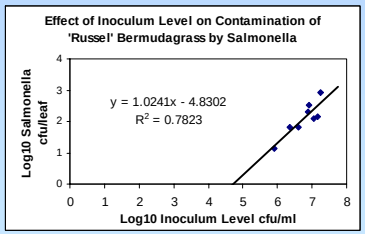
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	9.7888	7	1.3984	1.8671	0.0787	2.0703
Within Groups	113.84	152	0.749			
Total	123.63	159				

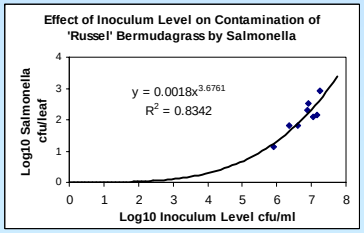


*inoculated on Russell, 10 leaves per isolate

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	3.1955	4	0.7989	0.8006	0.5312	2.5787
Within Groups	44.901	45	0.9978			
Total	48.097	49				





Linear model (top left) and power model (bottom left) of laboratory simulated contamination of Russell bermudagrass leaves with *Salmonella* R21 in WCW. Each point is the mean of 10 leaves. The models show a difference of about 2 logs in the predicted minimum inoculum level required for detectable levels of contamination. Future work will test these models with lower inoculum levels and refined detection and enumeration methods to improve estimates of the minimal *Salmonella* level required for detectable contamination of bermudagrass leaves.

Summary

Worst case water is a useful substitute matrix for swine lagoon effluent in controlled laboratory experiments with *Salmonella*.

No differences in levels of *Salmonella* contamination of bermudagrass leaves were observed among five *Salmonella* isolates tested in the laboratory simulation.

Laboratory simulated contamination of bermudagrass leaves with *Salmonella* varied widely (0 to 5x10^4 cfu/cm^2 leaf area) within and between eight cultivars tested.

Laboratory simulated contamination of bermudagrass leaves with *Salmonella* at measurable levels (> 5 cfu/cm^2 leaf area) required high levels (>10^6 cfu/ml) of *Salmonella* exposure in the worst case water matrix.

Laboratory simulation models predict that bermudagrass leaves in this system must be exposed to *Salmonella* levels >10^3 – 10^5 cfu/ml to produce detectable contamination.

Salmonella levels in all swine lagoons tested were below inoculum thresholds predicted in the simulation models and effluents from these lagoons would not be expected to produce measurable contamination of bermudagrass leaves.