WATER PATHOGENS PRESENT IN THE WASTEWATER FLOW OF A LARGE DAIRY IN THE SUMMER AND WINTER

C.W. Purdy, R.H. Raleigh, D.C. Straus, B.A Elmurodov

rom the Conservation and Production Research Laboratory, Renewable Energy, and Manure Management Research Unit, Agricultural Research Service, United States Department of Agriculture, P.O. Drawer 10, Bushland, Texas 79012 (Purdy); Veterinary Diagnostic Laboratory, Texas A&M University, Amarillo Blvd West, Amarillo, Texas 79109 (Raleigh); Department of Microbiology and Immunology, Texas Tech University Health Science Center, Lubbock, Texas 79430 (Straus); Uzbek Veterinary Research Institute, Taylak, Samarkand, Republic of Uzbekistan 704453 (Elmurodov).

The authors thank Will Willis for technical assistance.

Abstract

Dairy wastewater is a valuable resource in semi-arid regions that can be recycled, therefore it's potential content of epizootic pathogens, endotoxins and other hazardous substances is very timely and important. Bacterial and fungal epizootic pathogens were studied in wastewater on a 4,500 cow dairy in the Southern High Plains, U.S.A.. The wastewater was collected in triplicate from 13 different collection points along the wastewater drainage system, starting in the milking parlor and ending in a lagoon; and from an irrigation center pivot that applied the lagoon wastewater mixed with fresh water to forage crops, later used as feed for dairy cows. Pathogens were found at all collections points, except two points where chlorination was used. Eight *Salmonella* serovars were identified; most wastewater samples contained *Escherichia coll* O157:H7; and *Listeria monocytogenes* was found in mean concentrations from 1 x 10^4 to 1 x 10^5 CFU/ml. The mean concentrations of *Staphylococcus spp,* (1 x 10^4 to 1 x 10^7CFU/ml) and *Enterococcus spp* (1 x 10^4 to 10 x10^5 CFU/ml) were also high. The mean concentration of mesophilic fungi ranged from 1 x 10^2 to 1 x 10^5 CFU/ml. The center pivot wastewater contained fewer of each pathogen studied, and no *Enterococcus spp* were identified from this site. One of the most biologically active substances found in the lagoon wastewater was endotoxin which had a mean concentration of 37,712 EU/ml. These data show dairy wastewater can be a significant source of epizootic pathogens and endotoxin.

Introduction

Water is very precious in a semi-arid region and dairy wastewater is used as a valuable resource. The 30 year average precipitation in this region was 470 mm/year, and 30 year normal temperature in degrees centigrade was: maximum, 22, mean, 14, and minimum, 7. If this water contains dangerous pathogens there is the potential for contaminating larger areas of crops and pasture land. This then becomes a reservoir that can infect mammals and birds (Purdy, et al., 2001). Dairy wastewater that is used for dust control or for forage crops soon after irrigation, become problematic as a potential source of enteric pathogens.

Modem dairies in the U.S.A, have an ingenious way of using water to carry away much of the solid waste from the holding areas, milking parlors, and feed alleys located outside. The wastewater flows through ditches, and settling basins, before entering waste holding ponds (lagoons). The lagoon wastewater is often pumped through pipes to the irrigation center pivot irrigation systems, where it is usually blended with fresh well water prior to use.

The source of chlorinated water used to flush the fresh manure from the milking parlor floor was previously used to cool the large milk reservoir tanks. This same source of water is frequently used in sprinklers directed up to partially wash the cows udders of organic material while the cows wait in the holding area before they enter the milking parlor. The water used to flush manure from the long feeding alleys is recycled lagoon water.

The objective of this study was to determine the concentration of bacteria, and fungi, and to identify specific pathogen concentrations at 13 points along the flow of wastewater from the milking parlor to the lagoon and after pumping to the center pivot used for irrigation. In addition, the endotoxin concentration was determined at four lagoon sites.

Materials & Methods

Climate- The 30 year average precipitation in the Southern High Plains was 120.4 mm in the winter and 349.5 mm in the summer. The 30 year normal temperature in degrees centigrade for

the winter months (October through March) was: maximum, 15.1, mean, 7.5, and minimum, - 0.1; summer (April through September) was: maximum, 28.8, mean, 21.0, and minimum, 13.2. **Dairy-The** dairy was located in the Southern High Plains, where 4,500 head of cows were milked three times a day.

Sample collection for wastewater and **dilution** technique-Three-two-liter wastewater samples were collected in sterile plastic bottles from each of 13 points (milk parlor flush, holding area sprinklers, milk parlor effluent, beginning feeding alley flush, end feeding alley flush, beginning sand ditch, end sand ditch, settling basin, North lagoon, South lagoon, East lagoon, West lagoon, and center pivot). Each water sample was assayed for bacterial and fungal concentration. Briefly, 10-fold serial dilutions (W^{1} to $10'^{7}$) were prepared from each sample by inoculating 10 ml of wastewater into glass bottles containing 90 ml of sterile water. Aliquots of 0.1 ml/per dilution were spread over the surface of the Petri plates used in the dilution series for each medium used.

Culture medium - Specialized media were used to determine the concentration of bacteria and fungi, and to identify the pathogens (Brain-Heart-Infusion agar identified mesophilic and thermophilic bacteria[BHI], Difco; MacConkey agar was used for identifing all coliforms [MAC], Difco; Enterococcosel agar for identifing *Enterococcus spp.* [EGA], BBL; *Listeria* selective agar for identifing *Listeria monocytogenes.* [LSA], Oxoid; Bard Parker agar for identifing Gram-positive cocci; inducted were *Staphylococcus aureus, S. epidermidis, S. saprophyticus, and Micrococcus spp* [BPA],Oxoid; Malt Extract agar for identifing thermal fungi [ME], Difco; Littman oxgall agar for identifing mesophilic fungi [LOA], Difco. The fungi were further identified by colony formation, color and by spore identification (Crisan, EV, 1959; Barnett, HL., 1965; Larone, DH, 1995). The following inhibitory mediums (EC broth, Difco) were used for differentiating and enumerating *Escherichia coli* O157:H7, and a selenite-brilliant green- sulfapyridine enrichment broth medium (SBG plus Sulfa, Difco) was used in part for the isolation of *Salmonella spp.*

Additional specific Difco media (blood agar base supported with 5% bovine blood [BA] was used to help in the identification of hemolytic *Staphylococcus spp, Streptococcus spp, Corynebacterium spp,* and to determine that *Salmonella spp* were not hemolytic. In addition, Xylose-lysine-desoxycholate agar [XLD], Difco; Brilliant Green agar [BG], Difco; Lysine Iron agar [LIA], Difco; and Triple Sugar Iron agar [TSI], Difco were used to differentiate *Salmonella spp.* The *Salmonella spp* were serotyped by the National Veterinary Services Laboratory located at Ames, Iowa.

Culture incubation temperatures-Thermophilic bacteria (BHI medium) were incubated at 55°C, and thermophilic fungi (LOA medium) were incubated at 50°C. Pathogens were grown at 37°C, and mesophilic bacteria and fungi were incubated at 28°C.

Kinetic *Limulus* lysate assay-Endotoxin was measured by use of the kinetic chromogenic quantitative *Limulus* ameobocyte lysate assay (Williams and Halsey, 1997). Methods for the assay are fully described by Purdy et al. (2001).

Statistical analyses-Data were analyzed by use of ANOVA, using the general linear models procedure [a] (SAS user's guide, 1988). Significant differences between wastewater assay positions were further evaluated by use of the Bonferroni adjusted paired t-test. Differences were considered significant at $P < 0.05$.

Results

Eight *Salmonella* serotypes were identified in the winter and summer **(Table 1).** A total of 86 *Salmonella* isolates were identified, 6 *Salmonella* were untypeable, and 4 were multiple *Salmonella* serotypes. Sixty *Salmonella* suspected isolates were not *Salmonella,* which gave a 63% recovery rate. *Salmonella agona* was only found in the winter. *Salmonella spp* was found at 11 of the 13 collection sites, except in the milk barn flush water and the water from the milk barn holding area floor sprinklers.

Endotoxin mean concentration for the lagoon wastewater differed significantly with the seasons ($P > 0.007$), 49,800 (std error 3006) EU/ml in the winter and 25,624 (3992) EU/ml in the summer.

Escherichia coli O157:H7 was recovered from all EC enriched water samples, except those collected from the center pivot in the winter. The water from the origin of milk barn flush

and from the floor sprinklers were considered sterile, however occasional microbe contaminate were encountered due to the collection process.

The most numerous mesophylic fungal pathogens identified from the wastewater collection sites were *Mucor spp,* and *Aspergillus spp,* and the most numerous thermophilic fungi were *Mucor pusillus* and *Aspergillus fumigatus.*

The overall mean concentrations of bacteria and fungi found in the dairy wastewater collection areas are reported as colony forming units (CFU)/ml for selective media **(Table 2).** There were significant differences ($P < 0.045$) between the summer and winter and among the collection sites ($P < 0.004$) for mesophilic bacterial concentrations. There were no significant differences between mean concentration of thermophilic bacteria between seasons or among collection sites. There were significant differences between seasons and collection sites for the mean concentration of mesophilic fungi **(Table 2).** The mean concentrations of theromphilic fungi were determined only in the winter. At that time the mean concentrations were significantly different among collection sites ($P < 0.0001$).

Discussion

Dairy wastewater appears to be a significant source of enteric pathogens, *Salmonella spp,* and *Escherichia coli* O157:H7. In addition numerous Gram-positive pathogens such as S. *aureus, Streptococcus spp,* and *Corynebacterium spp* were isolated from the wastewater. *Listeria monocytogenes* was recovered most frequently. The endotoxin concentration of the dairy wastewater was high 37,712 (5121) EU/ml, but lower than that found in feedyard playas (8,321 ng/ml or 83,210 EU/ml) (Purdy, CW, et al., 2001).

Thermal fungi and thermal bacteria in wastewater were 100 times less frequent than mesophilic fungi and mesophilic bacteria at each collection site. (Data not shown). It is interesting that one to two log differences were seen at different sides of the lagoon using the selective media. This indicates that the lagoon microflora are not thoroughly mixed in the lagoon even under continuous multiple aerators.

The variety of bacterial pathogens and high levels of endotoxin should be considered before using this water for aerial spraying to abate dust. This practice would certainly increase environmental contamination, and increase the aerosol risk to animals and humans. The use of this untreated wastewater in the irrigation of plants that will be fed as forage without sustaining some drying (curing) period should be discouraged.

The spread of epizootic pathogens by dairy wastewater to other animals should be avoided. For example, in this study, eight *Salmonella* serovars were identified and many more serovars were probably present. We recommend that dairy wastewater used to settle dust be chlorinated to kill the pathogens present. It should be realized that the less organic material that the wastewater contains the more efficient chlorination will be. Additional animal research is needed to determine if the aerosolized endotoxin level induced by high pressure application of wastewater to settle dust is sufficient to lower milk production and induce fever in exposed livestock.

It appears that the wildlife most vulnerable to these wastewater pathogens would be migratory water fowl, which can gain access to the lagoon, regardless of fencing.

References

Barnett, H.L. 1965. Illustrated Genera of Imprefect Fungi, 2nd Ed., Burgess Publishing Co., Minneapolis, Minnesota.

Crisan, E.V. August 1959. The isolation and identification of Thermophilic Fungi. A Master of Science Thesis Submitted to the Faculty of Purdue University, Department, of Microbiology, Purdue Press,

Larone, D.H. 1995. Medically important fungi: A guide to identification, 3rd Ed., ASM Press, Washington, D C.

Purdy, C.W., Straus, D C., Parker, D.B., and Williams, B.P. 2001. Water quality in cattle feedyards playas in winter and summer. American Journal of Veterinary Research. 62:1402- 1407.

SAS *Users' Guide:Version 6.03 Edition.* 1988. Cary, NC: SAS Institute Inc.

Williams P.B. and Halsey, J.E. 1997. Endotoxin as a factor in adverse reactions to latex gloves. Annals of Allergy, Asthma, and Immunology 79:303-310.

Table 1. *Salmonella* serovars identified from dairy wastewater and the number of -each serovar isolated.

Salmonella serovars	Number isolated
agona	12
give	6
minnesota	14
montevideo	6
muenster	1
newport	10
typhimurium	36
Worthington	1
Untypeable Salmonella	4
Multiple Salmonella serotypes	6

Table 2. Mean microbial colorfy.forming units (CFU)/ml based on the dairy wastewater collection site and the selective media on which the wastewater dilutions were grown.

Collection site	Mean CFU/ml BHI	Mean CFU/ml LOA	Mean CFU/ml MAC	Mean CFU/ml BPA	Mean CFU/ml LSA	Mean CFU/ml ECA
Milk Parlor Effluent	1.35E+08 (8.96E+07)	1.62E+05 (9.10E+04)	1.65E+05 (6.92E+04)	4.08E+06 (1.82E+06)	5.97E+05 (1.60E+05)	6.37E+05 (4.73E+05)
Alley Flush Top	4.28E+07 (1.03E+07)	8.10E+05 (3.33E+05)	4.26E+04 (1.25E+04)	2.55E+06 (9.35E+05)	1.34E+05 (5.55E+04)	5.23E+04 (7.80E+03)
Alley Flush Bottom	2.66E+07 (9.51 E+06)	4.30E+05 (6.63E+04)	9.25E+04 (1.79E+04)	1.12E+07 (6.41 E+06)	1.19E+05 (8.73E+04)	7.26E+04 (3.09E+04)
Sand Ditch Top	5.53E+07 (1.94E+07)	2.06E+05 (9.30E+04)	2.38E+05 (7.12E+04)	6.33E+08 (2.45E+06)	2.34E+05 (7.14E+04)	9.38E+04 (1.45E+04)
Sand Ditch Bottom	3.33E+07 (4.32E+06)	3.80E+05 (1.71E+05)	1.51E+05 (5.10E+04)	6.69E+06 (2.47E+06)	2.26E+05 (6.31 E+04)	1.07E+05 (3.22E+04)
Collection site	Mean CFU/ml BHI[1]	Mean CFU/ml LOA[2]	Mean CFU/ml MAC[3]	Mean CFU/ml BPA[4]	Mean CFU/ml LSA[5]	Mean CFU/ml ECA[6]
Settling Basin	7.95E+07 (2.51 E+07)	3.76E+05 (1.74E+05)	3.61E+04 (1.38E+04)	2.04E+07 (1.09E+07)	2.14E+05 (5.57E+04)	1.26E+05 (5.62E+04)
Lagoon North	6.23E+06 (2.40E+06)	1.00E+05 (8.16E+04)	3.62E+04 (1.54E+04)	2.88E+06 (1.41 E+06)	1.70E+05 (1.23E+04)	1.84E+04 (7.52E+03)
Lagoon South	5.62E+06 (2.05E+06)	7.17E+02 (3.00E+02)	6.05E+04 (2.91 E+04)	8.47E+05 (2.92E+05)	1.33E+05 (1.52E+04)	7.97E+03 (3.56E+03)
Lagoon East	1.84E+07 (7.38E+06)	5.02E+04 (5.00E+04)	1.83E+05 (9.34E+04)	3.86E+06 (1.78E+06)	1.77E+05 (2.90E+04)	1.78E+04 (7.23E+03)
Lagoon West	1.19E+06 (3.05E+05)	4.37E+04 (2.29E+04)	7.06E+04 (2.17E+04)	5.80E+05 (7.29E+04)	1.26E+05 (1.82E+04)	6.72E+03 (1.97E+03)
Collection site	Mean CFU/ml BHI	Mean CFU/ml LOA	Mean CFU/ml MAC	Mean CFU/ml BPA	Mean CFU/ml LSA	Mean CFU/ml ECA
Center Pivot	3.68E+05 (3.39E+05)	2.54E+03 (1.83E+03)	6.33E+02 ' (3.31 E+02)	4.57E+02 (2.38E+02)	1.67E+00 (1.67E+00)	0.00E+00 (0.00E+00)

1. Brain heart infusion agar is a rich all purpose bacterial medium; 2. Littman oxgall agar specific for fungal isolation; 3. MacConkey agar specific for Gram-negative bacteria and followed EC enrichment broth which limited the growth to *Escherichia coli* and *E. coli* O157:H7; 4. Bard Parker selective media for *Staphylococcus spp* and *Micrococcus spp;* 5. *Listeria* selective agar for *Listeria monocytogenes;* Enterococcosel agar selective medium for *Enterococcus spp.*