Applications of Biotechnology in Today's Dairy Industry

S. P. Oliver, *PhD*
Department of Animal Science and the Food Safety Center of Excellence, The University of Tennessee, Knoxville, TN 37996

Abstract

Advances in biotechnology and molecular biology in the last decade or so have brought exciting new technologies that can/will be used to solve complex problems confronting animal agriculture. These advances could have a fundamental impact and perhaps revolutionize production agriculture systems as we now know them. For example, improved disease surveillance; enhanced disease resistance; increased animal growth, efficiency and productivity; and manipulation of food quality and quantity are just a few possibilities that could impact the agricultural sector in the not-too-distant future. The purpose of this communication is to describe how biotechnology and molecular biology are used in research on mastitis, food safety and foodborne pathogens, and whenever possible describe how these advances have/will impact food-producing animals with particular emphasis on the dairy industry.

Introduction

Much of the progress in the dairy industry has been due to advances in biological technology. For example, scientific feeding of cows, mechanical milking, genetic selection and artificial insemination of dairy cows, and discovery and implementation of mastitis control procedures are just a few technological advances that have benefited the dairy industry. The impact and magnitude of these advances are perhaps best appreciated when considering that total milk production in the US is greater today, with 50 percent fewer cows, than in the 1940s. This demonstrates quite clearly that biotechnological advances have had a profound impact on the dairy industry.

The definition of biological technology, or "biotechnology", varies depending upon who is using the term and the context of what is being described. Biotechnology is derived from "bio" meaning life or living systems, and technology, which is defined as a scientific method for achieving a practical purpose. Biotechnology is defined broadly as a collection of technologies that utilize biological or living systems of plant, animal or microbial origin, or specific compounds derived from these systems for the production of industrial goods and services. Another more succinct definition as defined in the American Heritage Dictionary is that biotechnology is the engineering and biological study of relationships between man and machines. To the molecular geneticist, biotechnology infers the transfer of cloned genes from one organism to the genetic makeup of another, which has also been termed genetic engineering. Regardless of the definition used, biotechnology is not new to the agricultural sector since man has been exploiting living systems for the production of food for centuries.

Molecular techniques such as the polymerase chain reaction (PCR), real-time PCR, multiplex PCR, restriction fragment length polymorphism, pulsed-field gel electrophoresis, ribotyping, single nucleotide polymorphism analysis, genomics, proteomics, DNA sequencing, and cloning are used more and more frequently in many research laboratories in the United States and throughout the world. Use of PCR-based techniques has facilitated the discovery of more effective methods for the detection of veterinary and foodborne pathogens associated with food-producing animal environments and foodborne pathogens causing disease in humans. These techniques have also been quite useful to delineate virulence factors as well as antimicrobial resistance genes of important veterinary and foodborne pathogens. Use of these techniques may facilitate the discovery of more effective methods for the prevention, control and detection of diseases affecting food-producing animals.

Advances in biotechnology and molecular biology in the last decade or so have brought exciting new technologies that can be used to solve complex problems. These advances could have a fundamental impact and perhaps revolutionize production agriculture systems as we now know them. Improved disease surveillance; enhanced disease resistance; increased animal growth, efficiency and productivity; manipulation of food quality and quantity; and increased plant growth and efficiency are just a few possibilities that could impact the agricultural sector. The purpose of this communication is to describe how biotechnology and molecular biology are used in research on mastitis, food safety and foodborne pathogens, and whenever possible describe how these advances have/will impact food-producing animals with particular emphasis on the dairy industry.

Examples of Biotechnological Advances Currently Used in the Dairy Industry

In the 1980s and early 1990s, much work was done on bovine somatotropin (bST) focusing primarily on enhancing milk production in an already established lactation. The bST gene was identified, cloned and transfected into expression vectors such as bacteria or yeast. Concomitantly, research on recombinant DNA technologies resulted in methods that provided large quantities of recombinant bST (rbST) that would be necessary for research purposes. Subsequent research demonstrated that rbST increased milk yield and efficiency of feed utilization in dairy cows.[38] A review of the effects of rbST on milk production, milk composition, dry matter intake and body condition score was carried out by an expert panel established by the Canadian Veterinary Medical Association.[3] A series of meta-analyses were used to combine data on production and nutrition-related parameters that were extracted from all randomized clinical trials, which had been published in peer-reviewed journals or which were provided by Health Canada, from the submission by Monsanto for registration of rbST in Canada. Recombinant bovine somatotropin was found to increase milk production by 11.3% in first-lactation cows and 15.6% in multiparous cows, although there was considerable variation from study to study. While some statistically significant effects on milk composition (percent fat, protein and lactose) were found, they were all very small. Treatment increased dry matter intake by an average 3.3 lb (1.5 kg)/day during the treatment period and dry matter intake remained elevated into the first 60 days of the subsequent lactation. Despite the increase in dry matter intake, treated animals had lower body condition scores at the end of the treatment period, and reduced scores persisted until the start of the subsequent lactation. Data from numerous studies have shown that rbST is galactopoietic and can significantly increase milk production after peak lactation. RbST is now utilized on many dairy operations throughout the US.

More recent work has evaluated the potential of bST for developing various management alternatives. One such approach is research being conducted on use of bST to shorten the dry period. Dry periods of six to eight weeks duration have been an industry standard, because shorter dry periods resulted in reduced milk yields in the subsequent lactation by 10 to 30%. However, more recent research has demonstrated no production losses for cows given a 30-day dry period.

A recent study evaluated effects of shortened or omitted dry periods on milk production for cows at mature-equivalent production (>26,400 lb [12,000 kg] of milk) and treated with bST.[1] Five multiparous and five primiparous cows from each farm were assigned to each treatment: 1) 60-day dry period, label use of bST (60DD); 2) 30-day dry period, label use of bST (30DD); 3) continuous milking, label use of bST (CMLST); and 4) continuous milking with continuous use of bST (CMCST). Per label, bST use started at 57 to 70 days-in-milk and ended 14 days before drying (60DD and 30DD) or expected calving date (CMLST). In primiparous cows, average milk yields during the first 17 weeks of lactation were reduced for cows on treatments 30DD, CMLST, and CMCST vs. the 60DD treatment. (84.3, 77.2, and 82.5 vs. 97.0 +/- 2.9 lb/day, respectively). For multiparous cows, respective milk yields did not differ (102.5, 95.5, 102.3, and 104.9 +/- 4.6 lb/day).

Shortened or omitted dry periods may impede mammary growth in primiparous cows, resulting in reduced milk yield in the subsequent lactation. In contrast, a shortened or omitted dry period with either bST protocol did not alter production in multiparous cows treated with bST. Quality aspects of prepartum milk and colostrum require additional characterization. For multiparous cows, milk income generated for short dry periods or for continuous milking might increase profitability. At 17 weeks of the subsequent lactation, estimates of the cumulative net margins of multiparous cows on the 30DD treatment and continuous milking treatments exceeded those of cows on the 60DD treatment by $40 to $60 per cow.[1]

Significant culling of high-producing cows with low fertility reduces profitability of dairy farms as those cows are replaced with heifers. Induced lactation of nonpregnant cows has been proposed as a management alternative to reduce culling and increase profits. A study by Magliaro *et al*[21] was conducted to evaluate the efficacy of bST to increase milk production in cows induced into lactation with estrogen plus progesterone, and to determine the profitability of inducing cows into lactation vs. using replacement heifers entering the herd as first-lactation cows. Parity 1 or greater, nonpregnant, healthy Holstein cows (n = 28) were induced into lactation by administration of estradiol-17 beta (0.075 mg/kg of body weight [BW] per day) and progesterone (0.25 mg/kg of BW per day) for seven days. Milking began on day 18. Cows were assigned randomly to control or bST treatment groups on day 37 +/- 20 of milking, and milk production was compared for 70 days. After the 70-day comparison, all cows received bST for the duration of lactation. Cows receiving bST produced more milk (62.5 lb [28.4 kg]/day) than controls (53.0 lb [24 kg]/day), with variable yields among cows. For the economic analysis, cows induced into lactation were compared to first-lactation cows in the same herd using fair market value for costs and multiple-component pricing for milk. Net present value for a cow induced into lactation was significantly greater ($1,966) than that for a first-lactation cow ($1,446). Results of this study suggest that bST

use in cows induced into lactation is profitable. If a reliable method were developed and approved by the US Food and Drug Administration, inducing nonpregnant cows into lactation could be used by dairy producers to increase profitability.[21]

Also in the late 1980s and early 1990s, much work was conducted on a vaccine that utilized a galactose 4-epimerase deficient R mutant of *Escherichia coli* (designated J5). Galactose 4-epimerase attaches oligosaccharides to gram-negative bacterial core antigens. Lack of this enzyme results in exposure of core antigens, which are immunogenic.[46] Immunization of cows with three doses of a whole-cell bacterin containing *E. coli* J5 plus Freund's incomplete adjuvant resulted in fewer cases of clinical coliform mastitis during the first three months of lactation. In addition, the vaccine was effective against several gram-negative pathogens including *E. coli*, *Klebsiella pneumoniae, Klebsiella oxytoca, Enterobacter aerogenes* and *Serratia marcesens*.[14] This vaccine is now used in many dairy herds in the United States as part of a mastitis prevention and control strategy during the dry period and during periods of mammary transition.

Genetic Engineering of Microorganisms

A. Lysostaphin: Lysostaphin is a cell wall degrading enzyme secreted by *Staphylococcus simulans* first described in 1965 that was shown to be bactericidal.[41] Lysostaphin lyses practically all known *Staphylococcus* species but is inactive against bacteria of other genera.[40] The lysostaphin gene was successfully cloned into *Bacillus subtilis* and *Escherichia coli*, resulting in production of recombinant lysostaphin.[15,40] Recombinantly derived lysostaphin was shown to be effective in the mouse and guinea pig against experimental *Staph. aureus* mastitis, indicating both prophylactic and therapeutic potential. However, this approach will most likely be of limited value since the foreign protein would be recognized as non-self, and after repeated administration antibodies would probably be made against the protein, thereby minimizing and perhaps negating beneficial effects.

B. Nisin: Nisin was first introduced commercially as a food preservative several years ago. The first established use was as a preservative in processed cheese products. Since then, numerous other applications in foods and beverages have been identified.[2] Nisin is currently recognized as a safe food preservative in processed cheese and various pasteurized dairy products in approximately 50 countries. Renewed interest is evident in the use of nisin in natural cheese production. Considerable research has been carried out on the antilisterial properties of nisin in foods, and a number of applications have been proposed. Uses of nisin to control spoilage lactic acid bacteria have been identified in beer, wine, alcohol production and low pH foods such as salad dressings. Further developments of nisin are likely to include synergistic action of nisin with chelators and other bacteriocins, and its use as an adjunct in novel food processing technology such as higher pressure sterilization and electroporation. Production of highly purified nisin preparations and enhancement by chelators has led to interest in the use of nisin for human ulcer therapy and mastitis control in cattle.[2]

C. Identification of Beta-Defensin Genes in Bovine Mammary Epithelial Cells: Defensins are a unique family of naturally occurring peptides that display cytotoxic and antimicrobial properties against gram-positive and gram-negative bacteria, mycobacteria, fungi and some enveloped viruses.[6] Group 1 beta-defensins are produced constantly in epithelial cells of kidney, salivary glands, respiratory tract, urogenital tract and placenta; group 2 beta-defensins are found in skin, tongue and urogenital tract where its expression is induced by the presence of microorganisms.[6] In cattle, beta-defensin was isolated initially from respiratory epithelial cells. Subsequently, 13 novel beta-defensins were purified from bovine neutrophils, and beta-defensins were identified in gastrointestinal, kidney and skin epithelial cells.

Recent work in my laboratory at The University of Tennessee detected beta-defensin genes in bovine mammary epithelial cells.[33,45] A primary bovine mammary epithelial cell culture, designated BTE, isolated from mammary ducts and a bovine mammary epithelial cell line (MAC-T) were used. Total chromosomal DNA was isolated from BTE and MAC-T cell monolayers. PCR fragments were generated using primers described previously and cloned. Inserted genes were sequenced and DNA sequences were blasted and compared using the National Center for Biotechnology Information database. Gene polymorphisms were analyzed by restriction enzyme digestion with *TaqI*, *MspI* and *HhaI*.

A 1.65 Kb PCR fragment and a 1.30 Kb PCR fragment were amplified from BTE and MAC-T bovine mammary epithelial cells, respectively. Fragments were cloned, sequenced and the resulting DNA sequence showed 99% homology with *Bos taurus* antimicrobial protein exon 1-2 (L13373), 96% homology with *Bos taurus* neutrophils (BNBD-5) beta-defensin gene (AJ278799), 93% homology with *Bos taurus* neutrophils (BNBD-4) beta-defensin gene (AF008307) and 92% homology with *Bos taurus* enteric beta-defensin gene (AF016539). The restriction enzyme polymorphism of amplified genes showed a similar pattern to genes described above. The DNA sequence as well as restriction enzyme patterns of PCR fragments obtained from mammary gland epithelial cells were similar to those de-

scribed for *Bos taurus* neutrophil and enteric beta-defensin genes. Because of epithelial origin and inducible-secretory nature of these peptides, we hypothesize that bovine mammary glands secrete beta-defensins onto their surface upon infection and therefore serve as innate defense against bacterial colonization. Preliminary studies with synthetic beta-defensin peptides have shown significant *in vitro* inhibition of mastitis and foodborne pathogens.[33]

Development of Transgenic Livestock

The first successful experiment on the transfer of cloned genes from one animal species to the genetic makeup of another was reported in the early 1980s when a cloned rabbit beta-globin gene was microinjected into the pronuclei of several hundred mouse eggs.[49] Another highly publicized genetic engineering study was reported in the December 1982 issue of *Nature* that showed the picture of a "supermouse" that had been cloned with the structural gene for rat growth hormone that resulted in a mouse that was about twice the size of normal mice.[35] While gene transfer has been successfully accomplished in mice, sheep, pigs and cows, the frequency of transgene incorporation and subsequent expression of the gene product is generally quite low. However, results suggest that current biotechnological methodologies can be used in livestock. Development of improved methods for constructing the foreign fusion gene, microinjection of genes into the pronucleus of single cell ova, successful implantation into surrogate mothers, development of embryos to term, demonstration that the foreign gene has been stably and heritably incorporated into the DNA of newborns, and proving that the gene is regulated to function in its new environment, will likely result in an explosion in "Molecular Pharming."[47,48]

One objective of "Molecular Pharming" is to produce pharmaceuticals for treating human diseases. Mammary glands of dairy cows are an ideal organ for producing complex bioactive molecules that can be harvested and purified. In the last decade, approximately a dozen companies have been created to capture the US market for pharmaceuticals produced from transgenic bioreactors estimated at $3 billion annually. Several products produced in this way are now in human clinical trials.[50] Another research approach is genetic engineering of the bovine mammary gland to alter the composition of milk for human consumption, such as increasing or altering endogenous proteins, decreasing fat and altering milk composition to resemble that of human milk. Initial studies using transgenic mice to investigate the feasibility of enhancing manufacturing properties of milk have been encouraging.[50]

Another exciting approach has been to enhance disease resistance via prokaryotic gene expression in eukaryotes. Research at USDA and at the University of Vermont has been done with a bacterial protein, lysostaphin. Lysostaphin is a peptidoglycan hydrolase normally produced by *Staph. simulans*. When the native form is secreted by transfected eukaryotic cells, it becomes glycosylated and inactive. However, removal of two glycosylation motifs through engineering asparagine to glutamine codon substitutions enables secretion of Gln(125,232)-lysostaphin, a bioactive variant. Three lines of mice were developed that produced varying levels of lysostaphin in milk in which the 5'-flanking region of the ovine beta-lactoglobulin gene directed the secretion of Gln(125,232)-lysostaphin into milk. Resistance to a *Staph. aureus* intramammary challenge was observed, and the highest-expressing mouse line was completely resistant.[20] Milk protein content and profiles of transgenic and nontransgenic mice were similar. These results demonstrate the potential of genetic engineering to combat a prevalent mastitis pathogen.[20]

Expanding this concept to cows, these investigators successfully linked a cloned lysostaphin gene to beta-lactoglobulin, a protein normally found in cows' milk, in an attempt to enhance resistance of dairy cows to *Staph. aureus* by enabling cells of the mammary gland to secrete additional antibacterial proteins. Transgenic cows secreting lysostaphin at concentrations ranging from 0.9 to 14 mg/ml in their milk were produced.[51] *In vitro* assays demonstrated the milk's ability to kill *Staph. aureus*. Three transgenic and 10 non-transgenic cows were challenged by intramammary inoculation of *Staph. aureus*. Increases in milk somatic cells, elevated body temperatures and induced acute phase proteins, all indicative of infection, were observed in all of the nontransgenic cows but in none of the transgenic animals. Protection against *Staph. aureus* mastitis appears to be achievable with approximately 3 mg/ml of lysostaphin in milk. These results are encouraging and indicate that genetic engineering can provide a viable tool for enhancing resistance to disease and improve the well being of livestock. Further experimentation with transgenics could result in targeted expression of several different microbicidal molecules into milk that would ultimately have a profound impact in the prevention and control of mastitis.[51]

The potential profitability of "Molecular Pharming" seems clear. However, the high cost of producing transgenic cattle and obstacles including low rates of gene integration, poor embryo survival and unpredictable transgene behavior are significant limitations to realizing the potential of transgenic cattle.[50] Obviously, these obstacles need to be overcome before transgenic technology will be introduced on a large scale to production agricultural sectors. However, the fact that transgenic introduction has been successfully accomplished in livestock and passed to subsequent genera-

tions is enough encouragement for molecular geneticists to continue the quest to unravel the mysteries of gene regulation and expression. It is clear that insertion of new genetic material into agriculturally important animals is feasible, but this approach will require extensive evaluation of the transgene and transgene product in model systems.[19] The theoretical possibilities appear endless. Further research could lead to overexpression of proteins produced by bovine mammary secretory epithelial cells such as lactoferrin that can influence growth of mastitis pathogens, integration and subsequent expression of cytokines and/or antibodies by epithelial cells that could enhance lymphocyte and neutrophil function, and prokaryotic genes expressed in an active form in eukaryotes such as antimicrobial peptides. These are but a few possibilities that could have an impact on bovine mastitis. Other potential benefits of disease resistant transgenic food-producing animals would be reduced use of antimicrobials, fewer problems associated with antibiotic residues in foods to be consumed by humans and less antimicrobial resistance developed in pathogens capable of causing human disease.

Cloning

Identifying host mechanisms that contribute to mastitis resistance is difficult due to variability observed with an outbred population. Progress towards identifying these mechanisms could be made more quickly with cows that are genetically similar. New techniques such as cloning now offer a similar opportunity to mastitis researchers. A team of scientists at The University of Tennessee led by Drs. Lannett Edwards and Neal Schrick have successfully cloned Jersey dairy cows from mastitis-susceptible cows and mastitis-resistant cows.[39] The mastitis-susceptible cow UT3888 has been chronically infected with *Strep. uberis* for about seven lactations, in spite of numerous attempts to eliminate the infection. Some of the cloned heifers have calved and are currently lactating, while others are of breeding age. (http://animalscience.ag.utk.edu/utcloneproject/). By having a unique set of genetically identical animals, it is possible to develop our understanding of what contributes to mastitis resistance or susceptibility under different management schemes, vaccination protocols, or stress-situations, without the added complication of genetic variation. Our first step towards identifying these mechanisms is to determine if differences in blood leukocyte profiles exist in comparison to age-matched herd-mates. Future research will be conducted to determine if immune responsiveness of clones from mastitis-susceptible animals are less than those of herdmates, thus contributing to susceptibility. Once identified, more basic research altering conditions of the entire animal can begin to dissect the mechanisms that contribute to susceptibility or resistance to mastitis or other diseases of dairy cattle. Identification of such factors could lead to improved selection strategies and/or novel approaches for eradicating or reducing incidence of mastitis and other diseases impacting dairy cows.

Identification of Disease-susceptible and Resistant Dairy Cows

Novel approaches are currently being developed and utilized to determine what genetic factors are involved in disease resistance. Identification of such factors will be critical for developing strategies for eradicating or reducing the incidence of disease. Selection of dairy cows for enhanced disease resistance without compromising production traits is a very appealing concept that, until the last decade, was primarily a theoretical fantasy. However, excellent molecular techniques have been developed resulting in the identification of new genetic markers that have been used to identify and characterize genes responsible for production traits and host immunity. Major histocompatibility complex (MHC) genes, also called bovine lymphocyte antigens or BoLA, have received much recent attention because of their involvement in host immunity. Significant associations have been made with some infectious diseases of cattle and BoLA genes. There is strong evidence indicating that BoLA genes are important in resistance or susceptibility to diseases such as mastitis, retained placenta and cystic ovarian disease in dairy cattle. For example, one BoLA-DRB gene pattern in a study of 106 Holstein cows was associated with resistance to *Staph. aureus* mastitis.

Results of our research on BoLA-DRB3.2 gene fingerprinting of Jersey cows at The University of Tennessee Dairy Experiment Station were published by Gillespie *et al.*[9] Jersey cows (n=172) were genotyped for the BoLA-DRB3.2 allele using PCR and restriction fragment length polymorphism analysis. Bovine DNA was isolated from aliquots of whole blood. A two-step PCR followed by digestion with restriction endonucleases *Rsa*I, *Bsty*I, and *Hae*III was conducted on the DNA from Jersey cattle. Twenty-four BoLA-DRB3.2 alleles were identified with frequencies ranging from 0.3 to 22.9%. Thirteen allele types were similar to those reported previously; eleven were new allele types that have not been reported. Allele types reported previously include: BoLA-DRB3.2*2, *8, *10, *15, *17, *20, *21, *22, *23, *25, *28, *36, and *37. Their frequencies were 0.3, 11.3, 22.9, 13.6, 5.5, 3.7, 10.7, 3.5, 0.9, 0.3, 4.7, 9.3, and 0.9%, respectively. Of the new allele types detected, *ibe occurred at the highest frequency (6.1%) in Jersey cows from this herd. The six most frequently isolated alleles (BoLA-DRB3. *8, *10, *15, *21, *36 and *ibe) accounted for about 74% of alleles in the population of this herd.

Results of our study demonstrated that the BoLA-DRB3.2 locus is highly polymorphic in Jersey cattle. Thus, the BoLA gene may not be the best candidate for determining a relationship between genotype and mastitis susceptibility or resistance in Jersey cows.

A genetic marker associated with inflammatory responses is also being evaluated. One potential marker is CXCR2, a chemokine receptor required for neutrophil migration to infection sites, which contains single nucleotide polymorphisms (SNP) within the gene. In a study by Youngerman *et al*,[52] single nucleotide polymorphisms (SNPs) and resulting haplotypes in the bovine CXCR2 gene were identified as a potential target for a genetic marker for mastitis susceptibility. A 311-bp segment of the bovine CXCR2 gene was amplified and sequenced. Five SNPs at positions 612, 684, 777, 858 and 861 were expressed in both Holstein and Jersey dairy cattle. Four SNPs resulted in synonymous substitutions, while a non-synonymous switch at position 777 (G to C) resulted in a glutamine to histidine substitution at amino acid residue 245. The five polymorphisms generated 10 distinct haplotypes. Six haplotypes were common between the two breeds, while Holsteins and Jerseys each uniquely expressed two haplotypes. Of the six common haplotypes, two represented 83% of the Jersey population, whereas four of these haplotypes represented 95% of the Holstein population.

The association of CXCR2 SNP genotypes with subclinical and clinical mastitis was evaluated by Youngerman *et al*.[53] Thirty-seven Holstein and 42 Jersey cows that completed at least two full lactations were used. A significant association was detected between CXCR2 SNP +777 genotype and percentages of subclinical mastitis cases in Holsteins. Holsteins expressing genotype GG had decreased percentages of subclinical mastitis, but genotype CC cows had increased percentages of subclinical mastitis. Significant differences in clinical mastitis incidence were not detected between genotypes for either breed. This approach of genetically identifying mastitis-resistant cows may represent an effective means of marker-assisted selection for mastitis and other inflammatory diseases involving neutrophils. The initial work is encouraging and several studies are ongoing in this exciting research area.

The *Streptococcus uberis* Story

Streptococcus uberis is an important cause of mastitis in dairy cows — particularly during the dry period, the period around calving and during early lactation — that is not controlled effectively by current mastitis control practices.[31] Many *Strep. uberis* intramammary infections (IMI) that originate during the non-lactating period and near calving result in clinical and subclinical mastitis during early lactation. Control programs for reducing *Strep. uberis* IMI should focus on periods adjacent to the non-lactating period where opportunities exist to develop strategies to reduce the impact of *Strep. uberis* infections in the dairy herd.[31]

We began our *Strep. uberis* research journey in the early 1990s. Earlier research in England demonstrated the presence of two *Strep. uberis* genotypes designated types I and II. Subsequent research from England determined the nucleotide sequences of 16S ribosomal RNA of *Strep. uberis* genotypes I and II and showed that the two genotypes were phylogenetically distinct, and proposed that *Strep. uberis* genotype II be designated *Streptococcus parauberis*. However, differentiation of *Strep. uberis* from *Strep. parauberis* was only possible by DNA hybridization or 16S rRNA sequencing, since cultural, morphological, biochemical and serological characteristics of the two closely related species are indistinguishable. A technique was developed by Jayarao *et al*[16] for differentiating *Strep. uberis* from *Strep. parauberis* based on DNA fingerprinting. Results of those studies demonstrated that the predominate organism isolated from infected mammary glands was *Strep. uberis* and that *Strep. parauberis* occurred infrequently. This method was also used for species identification and differentiation of bacteria of bovine origin. Using the PCR reaction, oligonucleotide primers complementary to 16S rRNA genes have been used to amplify the 16S ribosomal gene fragment from bacterial genomic DNA. Characteristic 16S rDNA fingerprint patterns have been used to correctly identify 11 different *Enterococcus* and *Streptococcus* species.[17]

Research from our laboratory has focused extensively on development of *in vivo* and *in vitro* models to study host-pathogen interactions, and on identification and characterization of virulence factors associated with the pathogenesis of *Strep. uberis* mastitis and other environmental streptococci.[31] We have shown that *Strep. uberis* was able to adhere to epithelial cells and that was followed by internalization into the host cell via exploitation of host cell machinery. Our lab demonstrated that *Strep. uberis* used host elements like extracellular matrix proteins to achieve increased adherence, probably utilizing these as a molecular bridge to attach to host cell membranes. Another of these host cell factors appears to be lactoferrin (LF), a whey protein found in milk. Use of molecular biology tools such as proteomics, genomics and bioinformatics has led to the discovery of a novel protein produced by *Strep. uberis* referred to as *Streptococcus uberis* Adhesion Molecule or SUAM.

We have conducted numerous studies on SUAM and a brief summary of these studies follows. Collectively, experiments from our laboratory have provided evidence that: 1) *Strep. uberis* produces SUAM;[4] 2) SUAM binds to LF in milk;[5] 3) binding of LF through SUAM enhances adherence of *Strep. uberis* to bovine

mammary epithelial cells.[5] Lactoferrin may function as a bridging molecule between *Strep. uberis* and bovine mammary epithelial cells, facilitating adherence of this important mastitis pathogen to host cells; 4) SUAM in the absence of LF influenced adherence to and internalization of *Strep. uberis* into bovine mammary epithelial cells; 5) SUAM was isolated, purified and sequenced; 6) a SUAM-like protein was identified in *Streptococcus dysgalactiae* subsp. *dysgalactiae* and *Streptococcus agalactiae*;[36] 7) SUAM-like proteins produced by *Strep. dysgalactiae* subsp. *dysgalactiae* bind to bovine LF similarly to what we observed with *Strep. uberis*;[37] 8) antibodies against SUAM (whole protein) and to a synthetic peptide (pepSUAM) encompassing 15 amino acids of the N-terminus of SUAM cross-reacted with homologous proteins present in other strains of *Strep. uberis,* demonstrating the ubiquity of SUAM across all strains of *Strep. uberis* evaluated; 9) pepSUAM and SUAM antibodies cross-reacted with *Strep. agalactiae, Strep. dysgalactiae* subsp. *dysgalactiae*, and *Streptococcus pyogenes*; and 10) antibodies directed against pepSUAM inhibit adherence to and internalization of *Strep. uberis* into bovine mammary epithelial cells, suggesting that pepSUAM is biologically active. In addition, we have determined the theoretical DNA sequence of SUAM and confirmed this by PCR and restriction digests. Further confirmation of the theoretical SUAM sequence was obtained when the SUAM gene from the mastitis pathogen *Strep. uberis* UT888 was amplified, cloned and sequenced. Sequence analysis demonstrated that UT888 SUAM has 99% sequence identity to the theoretical SUAM identified in the Sanger *Strep. uberis* genomic database by homology to the reverse translated peptide sequence. When the SUAM DNA sequence was compared to GeneBank (NCBI *nr* GeneBank), no homologies as an entire gene were found, demonstrating that SUAM is a unique *Strep. uberis* protein. We hypothesize that SUAM plays a critical role in the pathogenesis of streptococcal mastitis by facilitating bacterial adherence to bovine mammary epithelial cells. Our hypothesis is that *Strep. uberis* expresses SUAM and uses LF in milk and/or on the epithelial cell surface to adhere to mammary epithelial cells.

Nucleic Acid-based Methods for Mastitis Pathogen Detection

Rapid diagnosis of mastitis pathogens could enhance therapy and perhaps reduce destruction of secretory tissue and minimize subsequent milk loss. Microorganisms are currently differentiated utilizing defined media, an array of morphological, physiological, and biochemical characteristics and antibiotic susceptibility profiles. These procedures are labor intensive, expensive, and often require seven days or more to identify organisms. Simplified conventional identification schemes were developed, however, several days are still required to identify an isolate.

Detection and subtyping of bacteria for epidemiological evaluation has been made possible by randomly amplified polymorphic DNA (RAPD) fingerprinting. We have used this technique to identify *Streptococcus* species[7,11] and other mastitis pathogens,[18] and to detect new and persistent *Strep. uberis* and *Strep. dysgalactiae* subsp. *dysgalactiae* IMI in dairy cows.[32] Using phenotypic methods of streptococcal identification, these new IMI would not have been detected. RAPD fingerprinting has also been used for confirmation of *Strep. uberis* after intramammary challenge with *Strep. uberis* and identified new *Strep. uberis* infections in challenged quarters. Subtyping of *Strep. uberis* and *Strep. dysgalactiae* by RAPD fingerprinting demonstrated isolates from New Zealand were distinct from isolates from the United States.[8] RAPD fingerprinting has been used to study the possibility of *Staph. aureus* transmission by horn flies to heifers.[10,34] This technique is also useful in antibiotic efficacy studies in indicating new IMI or persistent IMI following antibiotic therapy.

Detection of Foodborne Pathogens and Virulence Factor Genes Using Different PCR Formats

The need for rapid, sensitive and reproducible techniques for bacterial strain identification is evident in many areas of public health, agriculture and national security. Bacterial detection methods for differentiating bacterial species and strains are based on both phenotype and genotype. Techniques based on phenotype, such as metabolic studies, serotyping and immunological methods, are not specific enough to completely distinguish among different genera, species and strains of bacteria and are not general enough to apply to a diverse set of pathogens. Additionally, genes may not be expressed under certain cultural conditions. Methods based on the genotype examine differences in DNA sequences and are much more successful in discriminating among different bacterial strains. The most definitive methods in use for bacterial subtyping are restriction fragment length polymorphism (RFLP), PCR-based methods and ribotyping. PCR-based methods require only small quantities of DNA, whereas RFLP requires relatively large amounts of DNA. Unique bacterial DNA-sequences (chromosomal and/or plasmidal) can be used for detection to the genus and in many cases to the species level.

Current methods used for routine identification and confirmation of foodborne pathogens such as *Campylobacter*, *Salmonella* spp, *Listeria monocytogenes* and Shiga toxin-producing *Escherichia coli* (STEC; O157

and non-O157 STEC) are generally slow, inadequate, laborious and non-existent in the case of critical detection of pathogens like non-O157 STEC. Many clinical laboratories do not routinely report non-O157 STEC serotypes since they cannot easily identify these microorganisms. Conventional diagnostic methods are often too cumbersome and time-consuming to be useful for timely monitoring of foods, especially those with limited shelf lives. Rapid detection methods could be used effectively for quality control in food processing facilities to rapidly screen incoming ingredients and raw materials. Rapid detection methods allow: (1) timely monitoring of food processing equipment and the immediate environment, (2) brisk corrective action (product recall and release of lots/batches of product for distribution), and (3) faster intervention in the case of threats of disease or potential death, without having to wait several days for results, as in the case of most current microbiological methods.

Shiga toxin-producing *E. coli* are of immense economic and public health significance. STEC O157:H7 are characterized by low infectious doses (1-100 colony-forming units) and are highly pathogenic in humans, where they cause serious acute illness and long-term sequelae. Manifestations of illnesses caused by STEC that are linked to production of Shiga toxins include non-bloody diarrhea, diarrhea-associated hemorrhagic colitis, hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura. Intimin and enterohemolysin are among the prominent ancillary virulence factors elaborated by STEC. There is a general consensus that ruminants are the main source of human pathogenic STEC. An array of food products that include beef, apple cider, salad, fruits and contaminated well water have been implicated in foodborne disease involving STEC. Sufficient evidence has been presented on the zoonotic nature of bacterial enteric pathogens and the role of companion animals as reservoirs of some human pathogenic STEC serotypes.

We used a multiplex PCR format to identify *E. coli* O157:H7 strains that target common virulence genes encoding Shiga toxins 1 and 2 (*stx*1 and *stx*2), enterohemolysin (hly_{933}), intimin (*eae*A) and flagellar H7 ($flicC_{h7}$) gene sequences.[22,23,24,26] The objective of one study was to characterize 400 *E. coli* isolates from dairy cows/feedlots, calves, mastitis, pigs, dogs, parrot, iguana, human disease and food products for prevalence of STEC virulence markers.[26] The rationale of the study was that isolates of the same serotype that were obtained from different sources and possessed the same marker profiles could be cross-species transmissible. Shiga toxin-producing isolates were tested for production of Shiga toxins (*stx*1 and *stx*2) and enterohemolysin. Of the *E. coli* O157:H7/H- strains, 150 of 164 (mostly human, cattle and food) isolates were *stx*-positive. Sixty-five percent of O157 STEC produced both *stx*1 and *stx*2; 32% and 0.7% produced *stx*2 or *stx*1, respectively. Ninety-eight percent of O157 STEC had sequences for genes encoding intimin and enterohemolysin. Five of 20 *E. coli* O111, four of 14 O128 and four of 10 O26 were *stx*-positive. Five of six *stx*-positive O26 and O111 produced *stx*1, however, *stx*-positive O128 were *stx*-negative. Acid resistance (93.3%) and tellurite resistance (87.3%) were common attributes of O157 STEC, whereas non-O157 *stx*-positive strains exhibited 38.5% and 30.8% of the respective resistances. *stx*-positive isolates were mostly associated with humans and cattle, whereas all isolates from mastitis (n=105), and pigs, dogs, parrots and iguanas (n=48) were *stx*-negative. Multiplex PCR was an effective tool for characterizing STEC pathogenic profiles, and distinguished STEC O157:H7 from other STEC. Isolates from cattle and human disease shared similar toxigenic profiles, whereas isolates from other disease sources had few characteristics in common with the former isolates. These data suggest interspecies transmissibility of certain serotypes, in particular, STEC O157:H7 between humans and cattle.

We have also used a multiplex PCR format to confirm and identify *Campylobacter jejuni* isolated from the dairy farm environment and from dairy cows.[25] *Campylobacter* is a leading cause of bacterial foodborne illness in the US and in many other industrialized countries. This organism is widespread in nature and can be isolated from the gastrointestinal tracts of many animal species, including poultry, freshwater and bulk tank milk. The ubiquity and low infectious dose of *Campylobacter* makes its presence in the food supply a significant health hazard. It is therefore important to have accurate and reliable methods for isolation and detection of *Campylobacter* spp, in particular *C. jejuni*, which is the most common species associated with acute bacterial enteritis. The major disadvantages of the commonly used phenotype-based typing schemes, such as biochemical tests, including serology, are that they are time-consuming, technically demanding and may lead to a high number of untypable strains. Consequently, there is an increasing need for highly sensitive and reliable DNA-based methods for typing *C. jejuni*. Targets we used for identification of *C. jejuni* were the hippuricase gene (*hip*) and a 23S rRNA gene specific for thermophilic *Campylobacter*. All 265 bulk tank milk samples analyzed were negative for *C. jejuni,* whereas five of 411 (1.2%) fecal samples tested positive. This is the first report that has used a combination of sequences of the two genes in a multiplex format to identify *C. jejuni* to the species level. The method described has potential for routine use in the detection of thermophilic *Campylobacter* in farm environmental samples as well as other samples. This multiplex PCR assay can decrease the time for identification and confirmation of *C. jejuni.*

Polymerase Chain Reaction-based Enzyme Linked Immunosorbent Assay (PCR-ELISA)

Molecular techniques can also be utilized to serogroup bacterial isolates. *Salmonella* are important foodborne pathogens that are responsible for serious cases of foodborne illness. *Salmonella* may be transmitted by a wide variety of agricultural products and processed foods. Foods of animal origin such as beef, pork, chicken, eggs and milk have been shown to carry these pathogens. Salmonellosis is commonly diagnosed in dairy cows and calves, and the presence of *Salmonella* on dairy farms has been well documented. Several serogroups of this bacterium occur with varying degrees of relevance to human and animal health. Identification of *Salmonella* is important for surveillance, prevention and control of foodborne diseases. An accurate and rapid procedure for identification of *Salmonella* is needed to identify sources, reservoirs, and transfer of these foodborne pathogens through the food chain. However, there are many problems associated with differentiating *Salmonella* species, subspecies and serovars. Current available screening tests only provide presumptive identification of *Salmonella* as a group without identification of serogroups. Negative results are considered definitive, but positive results must be confirmed by conventional methods and serology.

The concept of targeting gene sequences that encode for species specificity is promising. In *Salmonella*, the *rfb* gene clusters are responsible for biosynthesis of the O antigens of *Salmonella* lipopolysaccharide. Variations among different O antigen structures are manifested in the types of sugar present or arrangement of sugars. This variability provides the basis for serotyping *Salmonell*a into serogroups. This highly polymorphic *rfb* gene cluster has been targeted as a molecular marker for the organism for detection of *Salmonella* serovars. We used a polymerase chain reaction-based enzyme linked immunosorbent assay (PCR-ELISA) to identify *Salmonella* somatic groups B, C1, C2, D and E1.[12] Primers were selected from the *rfb* gene cluster, which is responsible for biosynthesis of O antigens of *Salmonella* lipopolysaccharide. Previously serogrouped *Salmonella* isolates (n=169) were evaluated by the PCR-ELISA procedure. DNA from all isolates was amplified using the PCR procedure for selected somatic groups and subjected to the ELISA procedure. This technique correctly identified 93% of *Salmonella* isolates belonging to somatic groups B, C1, C2, D and E1. The sensitivity of this procedure to correctly identify *Salmonella* somatic groups was 96% and the specificity was 98%. Utilization of this procedure circumvents the need to have *Salmonella* isolates serogrouped by state or regional reference laboratories.

Real-time PCR

We have dedicated much time and many resources attempting to develop real-time PCR techniques for detecting pathogens directly from milk. Real-time PCR is a relatively new DNA-based technique that monitors amplification of target DNA in real-time by monitoring florescence. Real-time PCR can be used to quantify bacteria from various samples including milk, feces, food and water. Real-time PCR can be used for processing, detecting and confirming pathogens in multiple samples at one time in a 96-well plate format. Additional post-detection methods are not utilized, therefore eliminating potential cross-contamination that may occur after amplification.

A multiplex real-time PCR method for simultaneous detection of *Staph. aureus*, *Strept. agalactiae* and *Strep. uberis* directly from milk has been developed.[13] These three mastitis pathogens frequently cause mastitis in dairy cows throughout the world. Targets we used for the multiplex real-time PCR were a *Staph. aureus*-specific genetic marker, the *cfb* gene encoding the Christie-Atkins-Munch-Petersen (CAMP) factor for *Strep. agalactiae,* and the plasminogen activator gene for *Strep. uberis*. Quarter milk samples (n=192) were analyzed by the multiplex real-time PCR assay and conventional microbiological methods. An additional 57 quarter milk samples were analyzed in a separate real-time PCR assay for *Strep. agalactiae* only. Using an overnight enrichment step, the real-time PCR technique correctly identified 96.4% of all quarter milk samples; 93% of *Staph. aureus,* 98% of *Strep. agalactiae* and 100% of *Strep. uberis*. Results of conventional microbiological methods were used to determine the sensitivity and specificity of the multiplex real-time PCR procedure. The sensitivity of this procedure to correctly identify *Staph. aureus*, *Strep. agalactiae* and *Strep. uberis* directly from milk was 95.5% and the specificity was 99.6%. Results of this study indicate that the multiplex real-time PCR procedure has the potential to be a valuable diagnostic technique for simultaneous identification of *Staph. aureus*, *Strep. agalactiae* and *Strep. uberis* directly from quarter milk samples. Manipulation of this multiplex real-time PCR method could be done to include additional or other frequently encountered pathogens found in milk, including foodborne pathogens.

We developed real-time PCR methods for identification of foodborne pathogens in food, dairy environmental samples and dairy cows. Real-time PCR assays utilizing dual labeled probes have been developed to identify *E. coli* O157:H7 and *L. monocytogenes* from beef products.[30] Target genes for *E. coli* O157:H7 and *L. monocytogenes* were *rfbE* and *hylA,* respectively. We have also used SYBR Green in real-time PCR assays to

detect *C. jejuni* from dairy farm environmental samples,[29] and a real-time PCR method utilizing SYBR Green I dye and a 119-bp fragment of the *invA* gene was evaluated for detection of *Salmonella* spp in dairy farm environmental samples.[28]

Development of diagnostic systems and rapid identification methods is progressing rapidly. Continued advances in rapid identification methods will undoubtedly result in development of assay systems capable of genus and species identification of mastitis pathogens on the farm. Increased sensitivity and accuracy of species level identification methods will aid epidemiological studies of mastitis pathogens.

Detection of Antimicrobial Resistance Genes in Veterinary and Foodborne Pathogens

Antimicrobials are used extensively in food-producing animals to combat disease and to improve animal performance. On dairy farms, antimicrobials such as tetracyclines, penicillins and sulfonamides are used to treat or prevent diarrhea and pneumonia, both of which are important diseases in dairy calves. Antimicrobials such as penicillins, cephalosporins, erythromycin and oxytetracyclines are used for treatment and prevention of mastitis, an important disease caused by a variety of gram-positive and gram-negative bacteria. Such drugs are often administrated routinely to entire herds to prevent mastitis during the non-lactating period. Benefits of antibiotic use in animal production systems include improved growth and/or feed efficiency, decreased nitrogen excretion and thus reduced environmental impact, decreased pathogen loads and a lower incidence of disease.

In contrast to the above benefits, however, are suggestions that agricultural use of antibiotics may be partly responsible for the emergence of antimicrobial resistant bacteria, which in turn may decrease the efficacy of similar antimicrobials used in human medicine. While investigations have focused on emergence of drug resistant bacteria, persistence of resistant bacteria and effects on human medicine, little information is available with regard to antimicrobial resistance of commensal bacteria and veterinary and foodborne pathogens on dairy production facilities, or management conditions that affect antimicrobial resistance. Information on prevalence of antimicrobial resistance, effects of stressors on the host animal, and the effect of management and environment at the farm level are especially lacking. Furthermore, much of the current available antimicrobial resistance data is derived from evaluating clinical isolates, originally obtained from sick animals. Consequently, this information may be biased by several factors, including housing or husbandry conditions, age and condition of animals tested, and previous antibiotic therapies. Because transferable resistance may originate from a variety of bacteria and associated hosts under a number of conditions, it is important that confounding factors are characterized so that more definitive conclusions can be derived.

The objective of our research is to gain insight into antimicrobial resistance gene flow from commensal bacteria of dairy farms to animal and human pathogenic bacteria. There is only limited information on rates and extent of gene exchange from commensal bacteria to animal and human pathogenic bacteria. We hope to define in detail the predominant resistance constructs in bacterial populations of dairy cows and their environment; and identify reservoirs, how antimicrobial resistance is transferred, and the relationships of antimicrobial use with development of antimicrobial resistance. Molecular tools such as DNA probes and PCR-based detection systems have greatly facilitated the study of the epidemiology of antimicrobial resistance genes and mobile genetic elements (MGE) and their transfer to other bacteria at the genetic level. Antimicrobial resistant commensal bacteria of dairy farms may play a pivotal role in the spread of antimicrobial resistance to pathogens that can cause disease in humans and animals. Since dairy cows are treated with many antimicrobial compounds for prevention and control of different diseases, commensal bacteria of cows and bacteria normally found in the dairy farm environment may acquire antimicrobial resistance.

We have used PCR to detect several different antimicrobial genes in a variety of veterinary and foodborne pathogens.[27,42,43,44] For example, in one study *C. jejuni* (n=39), *L. monocytogenes* (n=38) and *Salmonella* spp (n=12) isolated from dairy farms were evaluated for antimicrobial resistance gene patterns.[42] All foodborne pathogens were screened for 21 antibiotic resistance genes using PCR. *C. jejuni* (5.1%), *L. monocytogenes* (31.6%) and *Salmonella* spp (100%) contained more than one antibiotic resistance gene. Tetracycline resistant determinant (*tetA*) was found in 15.4, 31.6 and 100% of *C. jejuni*, *L. monocytogenes* and *Salmonella* spp, respectively; *tetC* was found only in *Salmonella* spp; and *tetB, tetC, tetE,* and *tetG* were not detected in any of the foodborne pathogens evaluated. The only other antimicrobial resistance gene detected in at least one isolate of each of the foodborne pathogens evaluated was *sulI*. A high frequency of *floR* (65.8%), *penA* (36.8%) and *strA* (34.2%) was found in *L. monocytogenes*. In *Salmonella* spp, *strA* (100%), *strB* (83.3%), *sulI* (100%), *ermB* (58.3%) and *penA* (50%) were amplified and all *Salmonella* spp were multi-drug resistant. Results of this study indicate that a high prevalence of foodborne pathogens isolated from the dairy farm environment contain antimicrobial resistance genes. The potential exists for foodborne pathogens carrying antimicrobial resistance

genes to acquire additional resistance genes as well as to spread this genetic material to commensal and pathogenic bacteria in the dairy farm environment.

In another study,[43] antimicrobial resistance gene patterns of 131 *E. coli* isolated from dairy cows with clinical mastitis were evaluated. All *E. coli* contained more than one antimicrobial resistance gene. Tetracycline resistance determinants, *tetA* and *tetC*, were found in 8.4% and 64.1% of isolates, respectively. Other tetracycline resistant determinants (*tetB*, *tetD*, *tetE* and *tetG*) were not observed in any of the isolates studied. Even though many *E. coli* carried the *tetC* gene, they were sensitive to tetracycline. Thus, tetracycline MIC data were negatively correlated with the presence of the *tetC* gene and positively correlated with the presence of *tetA* genes. Streptomycin resistance genes *strA* (7.6%) and *strB* (9.9%) and streptomycin-spectinomycin adenyltransferase gene (*aadA*) were found in 77.9% of test isolates. Ampicillin resistance gene (*ampC*) was the predominant gene (94.7%) found in *E. coli* from cows with mastitis. Over 99% of *E. coli* were resistant to ampicillin, and this correlated with the presence of the *ampC* gene. Other resistance genes, *penA* (49.6%), *sulI* (9.9%) and *sulII* (8.4%) were observed by PCR. Vancomycin resistance gene, *vanA,* was found in most *E. coli* (94.7%) but *vanB* was not present in any of the *E. coli* evaluated. Only one of 131 *E. coli* carried the *floR* gene. None of the isolates carried *cmlA*, *aac(3)IV*, *ermB*, *ereA* or *ereB*. In conclusion, all *E. coli* from cows with mastitis were multidrug resistant and carried more than one antimicrobial resistance gene. *E. coli* causing bovine mastitis may be a reservoir for antimicrobial resistance genes and may play a role in dissemination of antimicrobial resistance genes to other pathogenic and commensal bacteria in the dairy farm environment. However, further research is necessary to substantiate this hypothesis.

Conclusions

Advances in biotechnology and molecular biology in the last decade or so have brought exciting new technologies that can be used to solve complex problems. These advances could have a fundamental impact and perhaps revolutionize production agriculture systems as we now know them. For example, improved disease surveillance; enhanced disease resistance; increased animal growth, efficiency, and productivity; manipulation of food quality and quantity; and increased plant growth and efficiency are just a few possibilities that could impact the agricultural sector in the not-too-distant future.

Molecular techniques are very useful tools to study a variety of complex phenomenon and are used frequently in many research laboratories throughout the world. These techniques have been quite useful for identification of bacteria and subtyping of bacteria isolates for epidemiological applications, identification of genetic markers associated with disease susceptibility or resistance, and could aid in selection of dairy cattle that are more or less susceptible to mastitis. Use of PCR-based techniques have facilitated the discovery of more effective methods for the detection of foodborne pathogens associated with food-producing animal environments and foodborne pathogens causing disease in humans. These techniques have also been quite useful to delineate virulence factors and antimicrobial resistance genes of several important foodborne pathogens. Application of advances in biotechnology will allow dairy researchers greater flexibility to explore their area of scientific interest at the molecular level and may expedite discoveries leading to more effective methods for the control of mastitis and other diseases affecting dairy cows.

Recent and future biotechnological advances will undoubtedly impact the dairy industry and other sectors of production agriculture. As with any new technological development, thorough evaluation will be necessary to ensure accuracy, safety and efficacy. New technologies will likely place a greater emphasis on "good management" rather than result in a "magic bullet" approach to compensate for management inadequacies. We have pointed out just a few examples of some exciting technological advances that could have a significant impact on the prevention, treatment, and diagnosis of bovine mastitis. The future is promising and the theoretical possibilities of biotechnology appear endless. Only time will tell!

Acknowledgments

This work was supported by The University of Tennessee Food Safety Center of Excellence, the Tennessee Agricultural Experiment Station and The University of Tennessee College of Veterinary Medicine Center of Excellence Research Program in Livestock Diseases and Human Health. Authors express their appreciation to personnel in the Lactation/Mastitis/Food Safety Research Program at The University of Tennessee.

References

1. Annen EL, Collier RJ, McGuire MA, Vicini JL, Ballam JM, Lormore MJ: Effect of modified dry period lengths and bovine somatotropin on yield and composition of milk from dairy cows. *J Dairy Sci* 87:3746-3761, 2004.

2. Delves-Broughton J, Blackburn P, Evans RJ, Hugenholtz J: Applications of the bacteriocin, nisin. *Antonie Van Leeuwenhoek* 69:193-202, 1996.

3. Dohoo IR, Leslie K, DesCôteaux L, Fredeen A, Dowling P, Preston A, Shewfelt W: A meta-analysis review of the effects of recombinant bovine somatotropin1. Methodology and effects on production. *Can J Vet Res* 67:241-251, 2003.

4. Fang W, Oliver SP: Identification of lactoferrin-binding proteins in bovine mastitis-causing *Streptococcus uberis*. *FEMS Microbiol Lett* 176:91-96, 1999.
5. Fang W, Almeida RA, Oliver SP: Effects of lactoferrin and milk on adherence of *Streptococcus uberis* to bovine mammary epithelial cells. *Am J Vet Res* 61:275-279, 2000.
6. Ganz T: Versatile defensins. *Science* 298:977, 2002.
7. Gillespie BE, Jayarao BM, Oliver SP: Identification of *Streptococcus* species by randomly amplified polymorphic DNA fingerprinting. *J Dairy Sci* 80:471-476, 1997.
8. Gillespie BE, Jayarao BM, Pankey JW, Oliver SP: Subtyping of *Streptococcus uberis* and *Streptococcus dysgalactiae* isolated from bovine mammary glands by DNA fingerprinting. *J Vet Med* B 45:585-593, 1998.
9. Gillespie BE, Jayarao BM, Dowlen HH, Oliver SP: Analysis and frequency of bovine lymphocyte antigen DRB3.2 alleles in Jersey cows. *J Dairy Sci* 82:2049-2053, 1999.
10. Gillespie BE, Owens WE, Nickerson SC, Oliver SP: Deoxyribonucleic acid fingerprinting of *Staphylococcus aureus* from heifer mammary secretions and from horn flies. *J Dairy Sci* 82:1581-1585, 1999.
11. Gillespie BE, Oliver SP: Comparison of an automated ribotyping system, pulsed-field gel electrophoresis and randomly amplified DNA fingerprinting for differentiation of *Streptococcus uberis* strains. *Biotechnology* 3:165-172, 2004.
12. Gillespie BE, Mathew AG, Draughon FA, Jayarao BM, Oliver SP: A PCR-ELISA technique for detection of somatic group-specific *Salmonella* spp. *J Food Prot* 66:2367-2370, 2003.
13 Gillespie BE, Oliver SP: Simultaneous detection of *Staphylococcus aureus*, *Streptococcus agalactiae* and *Streptococcus uberis* in milk by multiplex real-time polymerase chain reaction. J Dairy Sci (Accepted), 2005.
14. Gonzalez RN, Cullor JS, Jasper DE, Farver TB, Bushnell RB, Oliver MN: Prevention of clinical coliform mastitis in dairy cows by mutant *Escherichia coli* vaccine. *Can J Vet Res* 53:301, 1989.
15. Heinrich P, Rosenstein R, Bohmer M, Sonner P, Gotz F: The molecular organization of the lysostaphin gene and its sequences repeated in tandem. *Mol Gen Genet* 209:563, 1987.
16. Jayarao BM, Dore Jr. JJE, Baumbach GA, Matthews KR, Oliver SP: Differentiation of *Streptococcus uberis* from *Streptococcus parauberis* by polymerase chain reaction and restriction fragment length polymorphism analysis of 16S ribosomal DNA. *J Clin Micro* 29:2774-2778, 1991.
17. Jayarao BM, Dore Jr. JJE, Oliver SP: Restriction fragment length polymorphism analysis of 16S ribosomal DNA of *Streptococcus* and *Enterococcus* species of bovine origin. *J Clin Microbiol* 30: 2235-2240, 1992.
18. Jayarao BM, Gillespie BE, Oliver SP: Application of randomly amplified polymorphic DNA fingerprinting for species identification of bacteria isolated from bovine milk. *J Food Prot* 59: 615-620, 1996.
19. Kerr DE, Wellnitz O: Mammary expression of new genes to combatmastitis. *J Anim Sci* 81 Suppl 3:38-47, 2003.
20. Kerr DE, Plaut K, Bramley AJ, Williamson CM, Lax AJ, Moore K, Wells KD, Wall RJ: Lysostaphin expression in mammary glands confers protection against staphylococcal infection in transgenic mice. *Nat Biotechnol* 19:66-70, 2001.
21. Magliaro AL, Kensinger RS, Ford SA, O'Connor ML, Muller LD, Graboski R: Induced lactation in nonpregnant cows: profitability and response to bovine somatotropin. *J Dairy Sci* 87(10):3290-3297, 2004.
22. Murinda SE, Nguyen LT, Ivey SJ, Gillespie BE, Almeida RA, Oliver SP: Prevalence and molecular characterization of *Escherichia coli* O157:H7 in bulk tank milk and fecal samples from cull dairy cows: a 12-month survey of dairy farms in East Tennessee. *J Food Prot* 65:752-759, 2002.
23. Murinda SE, Nguyen LT, Nam HM, Almeida RA, Headrick SJ, Oliver SP: Detection of Shiga toxin-producing *Escherichia coli*, *Listeria monocytogenes*, *Campylobacter jejuni*, and *Salmonella* spp. in dairy farm environmental samples. *Foodborne Path Dis* 1:97-104, 2004.
24. Murinda SE, Batson SD, Nguyen LT, Gillespie BE, Oliver SP: Phenotypic and genetic markers for serotype-specific detection of Shiga toxin-producing *Escherichia coli* O26 strains from North America. *Foodborne Path Dis* 1:125-135, 2004.
25. Murinda SE, Nguyen LT, Oliver SP: Problems in isolation of *Campylobacter jejuni* from frozen-stored raw milk and bovine fecal samples:genetic confirmation of isolates by multiplex PCR. *Foodborne Path Dis* 1:166-171, 2004.
26. Murinda SE, Nguyen LT, Landers TL, Draughon FA, Mathew AG, Hogan JS, Smith KL, Hancock DD, Oliver SP: Comparison of *Escherichia coli* isolates from humans, food, farm and companion animals for presence of Shiga-toxin producing *Escherichia coli* virulence markers. *Foodborne Path Dis* 1(3):178-184, 2004.
27. Murinda SE, Ebner PD, Nguyen LT, Mathew AG, Oliver SP: 2004e. Class 1 integrons, antimicrobial and acid resistance in pathogenic *Escherichia coli* isolates from dairy cow mastitis milk and feces. *Foodborne Path Dis* (Accepted), 2005.
28. Nam HM, Srinivasan V, Gillespie BE, Murinda SE, Oliver SP: Application of SYBR green real-time PCR assay for specific detection of *Salmonella* spp. in dairy farm environmental samples. *Int J Food Microbiol* (Accepted), 2005.
29. Nam HM, Srinivasan V, Murinda SE, Oliver SP: Specific detection of *Campylobacter jejuni* in dairy farm environmental samples using SYBR Green real time PCR. *Foodborne Pathogens & Disease* (Accepted), 2005.
30. Nguyen LT, Gillespie BE, Nam HM, Murinda SE, Oliver SP: Detection of *Escherichia coli* O157:H7 and *Listeria monocytogenes* in beef products by real-time polymerase chain reaction. *Foodborne Pathogens & Disease* 1 (4):231-240, 2004.
31. Oliver SP, Almeida RA, Calvinho LF: Virulence factors of *Streptococcus uberis* isolated from cows with mastitis. *J Vet Med* B 45:461-471, 1998.
32. Oliver, SP, Gillespie BE, Jayarao BM: Detection of new and persistent *Streptococcus uberis* and *Streptococcus dysgalactiae* intramammary infections by polymerase chain reaction-based DNA fingerprinting. *FEMS Microbiol Lett* 160:69-73, 1998.
33. Oliver SP, Tamilselvam B, Srinivasan V, Luther DA, Gillespie BE, Almeida RA: Identification of β-defensin genes in bovine mammary epithelial cells. *Proc Intl Dairy Fed Mastitis Conf* (In press), 2005.
34. Owens WE, Oliver SP, Gillespie BE, Ray CH, Nickerson SC: The role of horn flies (*Haemetobia irritans*) in *Staphylococcus aureus*-induced mastitis in dairy heifers. *Am J Vet Res* 59:1122-1124, 1998.
35. Palmiter RD, Brinster RL, Hammer RE, Trumbauer ME, Rosenfeld MG, Birnberg NC, Evans RM: Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes. *Nature* 300:611, 1982.
36. Park HM, Almeida RA, Oliver SP: Identification of lactoferrin-binding proteins in *Streptococcus dysgalactiae* subsp. *dysgalactiae* and *Streptococcus agalactiae* isolated from cows with mastitis. *FEMS Microbiol Lett* 207:87-90, 2002.
37. Park HM, Almeida RA, Luther DA, Oliver SP: Binding of bovine lactoferrin to *Streptococcus dysgalactiae* subsp. *dysgalactiae* isolated from cows with mastitis. *FEMS Microbiol Lett* 208:35-39, 2002.
38. Peel CJ, Bauman DE: Somatotropin and lactation. *J Dairy Sci* 70:474, 1987.
39. Pighetti GM, Edwards JL, Schrick FN, Saxton AM, Davies CJ, Oliver SP: Cloning adult dairy cows: a viable new tool in the fight against mastitis. *Proc Natl Mastitis Counc* pp 360-361, 2003.
40. Recsei PA, Gruss AD, Novick RP: Cloning, sequence, and expression of the lysostaphin gene from *Staphylococcus simulans*. *Proc Natl Acad Sci (USA)* 84:1127, 1987.
41. Schindler CA, Schuhardt UT: Purification and properties of lysostaphin-A lytic agent for *Staphylococcus aureus*. *Biochim Biophys Acta* 97:242, 1965.
42. Srinivasan V, Nam HM, Nguyen LT, Tamilselvam B, Murinda SE, Oliver SP: Prevalence of antibiotic resistance genes and integrons in foodborne pathogens isolated from dairy farms. *Proc Natl Mastitis Counc* pp 369-370, 2004.

43. Srinivasan V, Gillespie BE, Nguyen LT, Lewis MJ, Schukken YH, Oliver SP: Phenotypic and genotypic antibiotic resistance patterns of *Escherichia coli* isolated from dairy cows with mastitis. *Proc Natl Mastitis Counc* pp 371-372, 2004.
44. Srinivasan V, Tamilselvam B, Nam HM, Nguyen LT, Oliver SP: Prevalence of antibiotic resistant bacteria and antibiotic resistance genes in soil from dairy farms. *Proc Natl Mastitis Counc* pp 373-374, 2004.
45. Tamilselvam B, Srinivasan V, Luther DA, Almeida RA, Oliver SP: Identification of b-defensin genes in bovine mammary epithelial cells. *Proc Natl Mastitis Counc* pp 367-368, 2004.
46. Tyler JW, Cullor JS, Osburn BI, Bushnell RB, Fenwick BW: Relationship between serologic recognition of *Escherichia coli* 0111:B4 (J5) and clinical mastitis in cattle. *Am J Vet Res* 49:1950, 1988.
47. Van Brunt J: Bringing biotech to animal health care. *Bio/Tech* 5:677, 1987.
48. Van Brunt J: Molecular farming: Transgenic animals as bioreactors. *Bio/Tech* 6:1149, 1988.
49. Wagner TE, Hoppe PO, Jollick JD, Scholl DR, Hodinka RL, Gault JB: Microinjection of a rabbit beta-globin gene into zygotes and its subsequent expression in adult mice and their offspring. *Proc Natl Acad Sci (USA)* 78:6376, 1981.
50. Wall RJ, Kerr DE, Bondioli KR: Transgenic dairy cattle: genetic engineering on a large scale. *J Dairy Sci* 80:2213-2224, 1997.
51. Wall RJ, Powell AM, Paape MJ, Kerr DE, Bannerman DD, Pursel VG, Wells KD, Talbot N, Hawk HW: Genetically enhanced cows resist intramammary *Staphylococcus aureus* infection. *Nat Biotechnol* 23:445-51, 2005.
52. Youngerman SM, Saxton AM, Pighetti GM: Identification of single nucleotide polymorphisms, haplotypes and their frequencies within the bovine IL-8 receptor locus in Jersey and Holstein cattle. *Immunogenetics* 56:355-359, 2004.
53. Youngerman SM, Saxton AM, Oliver SP, Pighetti GM: Analysis of bovine CXCR2 polymorphisms with subclinical and clinical mastitis incidence in Holstein and Jersey cattle. *J Dairy Sci* 87:2442-2448, 2004.