BARD

FINAL REPORT

PROJECT NO. US-2648-95

Identification of *Staphylococcus aureus* Virulence Factors Associated with Bovine Mastitis

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Project Title: Identification of *Staphylococcus aureus* Virulence Factors Associated with Bovine Mastitis

Investigators

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Keywords *not* appearing in the title and in order of importance. Avoid abbreviations.
Mammary, Vaccine, Staphylococcal enterotoxins, leukocidin, phosphotransferase

Abbreviations commonly used in the report, in alphabetical order:

Budget: IS: \$129,6000

US: \$170,400

Total: \$300,000

Signature
Principal Investigator

Signature
Authorizing Official, Principal Institution

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Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted)		3	2	5
Submitted, in review, in preparation		2	3	5
Invited review papers				
Book chapters				
Books				
Master theses				
Ph.D. theses		1		1
Abstracts		2		2
Not refereed (proceedings, reports, etc.)		1		1

Postdoctoral Training: List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant.

Jane Maddox (380-72-0621)

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings		2	1	3
Longer Visits (Sabbaticals)				

Description of Cooperation:

Both research groups extensively characterized *S. aureus* isolates using phenotypic and genetic parameters. The teams work cooperatively in identifying the most virulent isolates and specific surface associated and secreted virulence factors. Scientists from both countries assessed the affects of uniquely expressed virulence factors on important host defense mechanisms.

Patent Summary (numbers)

	Israeli inventor (s) only	US inventor (s) only	Joint IS/US inventors	Total
Submitted				
Issued (allowed)				
Licensed				

ABSTRACT

Identification of *Staphylococcus aureus* Virulence Factors Associated with Bovine Mastitis

Staphylococcus aureus is a major cause of mastitis in dairy cattle. The organism is able to adhere to and penetrate mammary epithelium, forming deep seated abscesses that result in chronic infections. This study was based on the observation that certain genotypes of *S. aureus* are isolated more frequently from field cases of bovine mastitis than others and the most prevalent genotypes of *S. aureus* have an increased ability to resist neutrophil phagocytosis and killing compared to the rare variants. It was hypothesized that these predominating genotypes differentially express virulence factors that allow them to overcome or suppress essential host defense mechanisms and successfully colonize mammary parenchyma. The overall objective of this study was to determine the mechanisms by which predominating *S. aureus* genotypes were able to resist mammary gland defense mechanisms. The following specific aims were accomplished to address the overall objectives of this project:

1. Analyze and compare cell surface and secreted protein profiles of common and rare *Staphylococcus aureus* genotypes isolated from field cases of bovine mastitis.
2. Purify and sequence selectively synthesized proteins unique to the most prevalent genotypes of *Staphylococcus aureus*.
3. Determine the *in vitro* effects of isolated proteins on essential host defense mechanisms.

Results from each specific aim showed that these predominating genotypes differentially express factors that may allow them to overcome or suppress essential host defense mechanisms and successfully colonize mammary parenchyma. Using complementary approaches, both the US and Israeli teams identified differentially expressed *S. aureus* factors that were positively correlated with virulence as determined by the ability to modify host immune cell responses and increase disease pathogenesis. Several candidate virulence factors have been identified at both the molecular (US team) and protein (Israeli team) levels. Components of the phosphotransferase system were shown to be differentially expressed in prevalent strains of *S. aureus* and to modify the growth potential of these strains in a milk microenvironment. Evidence provided by both the Israeli and US teams also demonstrated a potential role of Staphylococcal enterotoxins in the pathogenesis of mastitis. Certain enterotoxins were shown to directly affect neutrophil bactericidal activities which can profoundly affect the establishment of new intramammary infections. Other evidence suggests that *S. aureus* superantigens can suppress mammary defenses by enhancing lymphoid suppressor cell activity. Collectively, these data suggest that unique factors are associated with predominating *S. aureus* genotypes that can affect *in vitro* and *in vivo* virulence as related to the pathogenesis of bovine mastitis. The potential development of a subunit mastitis vaccine which incorporates only relevant antigenic determinants has not been investigated in depth. Experiments outlined in this proposal has identified putative virulence factors which contribute to the pathogenesis of *S. aureus* mastitis and which may be used to formulate an efficacious subunit mastitis vaccine. Results from these studies may lead to the development of new methods to prevent this costly disease, providing a viable alternative to less effective mastitis control procedures based on chemotherapy.

ACHIEVEMENTS

Significance of Achievements:

The first year of this project focused on analyzing and comparing cell surface and secreted proteins profiles of common and rare *Staphylococcus aureus* genotypes isolated from field cases of bovine mastitis. These studies were completed by phenotypic and genotypic characterization of strains from both countries. The phenotypic characterization of field isolates suggest that differences in protein expression patterns were apparent between 50 to 36 kDa as determined by gel electrophoresis. No correlation between any combination of the phenotypic characteristics was found when correlations were done with milk yield and somatic cell count. However, a positive correlation was found between type of haemolysis and the N-acetyl- β -D-glucosaminidase (NAGase) values. The genotypic characterization of strains resulted in the ability to subtype strains into 40 different genotypes by restriction fragment length polymorphism of the coagulase gene. These results indicated that the profiles of coagulase genotype differ among geographic locations, and that only a few genotypes prevail in each location. In addition, the predominant genotypes were more resistant to neutrophil bactericidal activities than rare genotypes.

Based on these initial findings, the next logical step was to focus on the identification and sequencing of unique virulence factors from selected strains obtained from both countries. The American group compared the gene composition of rare and prevalent strains of *S. aureus* using differential display analysis. To this end, a novel technique was developed to enrich mRNA from total prokaryotic RNA as a preparative step for differential display techniques. Results from these studies revealed several uniquely expressed transcripts from the prevalent genotypes including key components of the phosphotransferase system. In similar studies, multiplex PCR was then used to detect enterotoxin genes amongst the genotype subgroups in order to determine their possible role as virulence candidates that enable strains to combat neutrophil killing. The prevalent genotype strains were found to possess higher production capabilities for enterotoxin A as compared to low prevalence strains. The significance of enterotoxin A production capabilities in affecting pathogenicity of *S. aureus* strains was evaluated and found to have a profound effect on neutrophil killing abilities.

Additional studies were conducted in an attempt to determine the *in vitro* and *in vivo* effects of

isolated proteins on essential host defense mechanisms. The role of *S. aureus* exosecretions in mastitis was tested by intramammary inoculation of the isolated proteins and monitoring host responses. An association between two proteins in the exosecretion preparations and the severity of the cow's response to challenge. These findings suggest that Staphylococcal enterotoxins are responsible for, or associated with, bacterial penetration and colonization of the mammary gland. Staphylococcal enterotoxins with superantigen activity could play a role in the severity and duration of disease. Evidence suggests that *S. aureus* superantigens can suppress mammary gland resistance mechanisms by enhancing suppressor cell activity during disease pathogenesis.

Collectively, these studies illustrate the utility of a large epidemiological database as a tool for subgrouping strains with varying degrees of pathogenicity. This has allowed for the identification of relevant and previously undefined virulence factors that affect the ability of *S. aureus* to overcome innate and specific host defenses mechanisms. The potential development of a subunit mastitis vaccine which incorporates only relevant antigenic determinants has not been investigated in depth. Results from these studies may lead to the development of new methods to prevent this costly disease, providing a viable alternative to less effective mastitis control procedures based on chemotherapy.

Details of Cooperation:

Data generated from this project was a result of a coordinated effort between the US and Israeli groups. Isolates of *S. aureus* were characterized extensively by both phenotype (Israeli Team) and genotype (American Team). A representative from the Kimron Institute (Dr. Ahmad Younis) traveled to Penn State at the end of the first year to share research results of the *S. aureus* phenotyping and to learn the coagulase genotyping methodology. At this time, arrangements to share bacterial isolates were arranged. Dr. Gabriel Leitner also visited Penn State in the second year to compare data and strategies for the final year of the project. Based on the findings of both groups, a strategy was developed to proceed with the next series of experiments to identify unique virulence factors associated with the most prevalent strains of *S. aureus*. For this purpose, select strains from both the Israeli and American collection were identified for differential display analysis during the final years of the project. All research objectives were accomplished and both research teams assisted in the preparation of the final report.

List of Publications:

1. Su, C. and L.M. Sordillo. 1998. A simple method to enrich mRNA from total prokaryotic RNA. *Molecular Biotechnology*. 10:83-85.
2. Su, C., C. Herbelin, and L.M. Sordillo. 1999. Coagulase gene polymorphism of *Staphylococcus aureus* isolates causing bovine mastitis in different geographical areas. *Epidem. Infection*. 122:329-336.
3. Younis, A., G. Leitner, D.E. Heller, Z. Samra, R. Gadba, G. Lubashevsky, M. Chaffer, N. Yadlin, M. Winkler, and A. Saran. 2000. Phenotypic characteristics of *Staphylococcus aureus* isolated from bovine mastitis in Israeli dairy herds. *J. Vet. Med. B*. 47:591-598.
4. Leitner, G., N. Yadlin, A. Glickman, M. Chaffer, and A. Saran. 2000. Systemic and local immune response of cows to intramammary infection with *Staphylococcus aureus*. *Res. Vet. Sci*. 69:181-184.
5. Mullarky, I.K., C. Su, N. Frieze, Y.H. Park, and L.M. Sordillo. 2001. *Staphylococcus aureus* *agr* genotypes with enterotoxin production capabilities can resist neutrophil bactericidal activity. *Infect. Immun*. 69:45-51.

APPENDIX

Published Papers

Molecular Biotechnology.	A1
Epidem. Infection.	A2
J. Vet. Med.	A3
Res. Vet. Sci.	A4
Infection & Immunity.	A5

Submitted Papers

Leitner et al., J. Dairy Res.	A6
Younis et al, J. Dairy Res.	A7
Leitner et al., J. Dairy Sci.	A8

Unpublished data

The 4 different Staphylococcal exotoxins appearing between 36 kDa to 31 kDa in a one dimensional SDS-PAGE were purified and microsequenced. Two proteins were identified as leukocidin (LukKF-PV-chain and lukM procurer).

Sequence information.	A9
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