Population and Virulence Factor Analysis of *Staphylococcus aureus* from Bovine Mastitis

Fitzgerald, J.R., Meaney, W.J., Hartigan, P.J., and Smyth, C.J.

Department of Microbiology, Moyne Institute of Preventive Medicine, Trinity College, Dublin.

Department of Physiology, Trinity College, Dublin.

Teagasc acknowledges with gratitude the support of Structural Funds in the financing of this research project.

Project No. 4407

Teagasc,
19 Sandymount Avenue,
Ballsbridge,
Dublin 4.
November, 2000

CONTENTS

SUMMARY	3
INTRODUCTION	7
Typing of bovine *S. aureus* isolates using RAPD PCR	9
Typing of multiple isolates from the same primary isolation plate	10
Typing of multiple isolates from the same herd	11
Comparison of RAPD PCR to other molecular typing techniques	12
Virulence factor analyses	13
Characterisation of pathogenicity island (SaPI1)	14
In-vitro superantigens functional analysis	14
Mouse mastitis model	15
Rabbit infection model	15
CONCLUSIONS	16
PUBLICATIONS	18

SUMMARY

Staphylococcus aureus is a major cause of bovine mastitis and the disease is responsible for substantial economic losses in the dairy industry world-wide. A large number of commonly accepted virulence factors are associated with *S. aureus* but it is yet to be elucidated which of these are important for infection of the bovine udder.

A rational and effective strategy for the control of intramammary infections may need to be directed against clones of *S. aureus* that commonly cause disease. The objective of this study was to characterise the genetic variance of *S. aureus* isolate populations from infected udders in Ireland using RAPD-PCR, ribotyping and multilocus enzyme electrophoresis (MLEE). Similar *S. aureus* isolates collected in the USA were also typed in order to compare strain differences in staphylococcal populations in a different environment. Phenotypic diversity based on a number of presumed virulence factors together with antibiotic sensitivity was examined and correlations between phenotype and genotype were identified, if present. In addition, a pathogenicity island encoding multiple superantigens was completely sequenced and characterised. Knockout mutants of these superantigens were also constructed and *in vitro* functional analysis performed.

Laboratory animal experiments (mice and rabbits) were used to study the relative pathogenicity of individual staphylococcal strains (mice) and also to measure the immunological responses after prolonged exposure to the predominant strains (rabbits).

The results may be summarised as follows :

- RAPD-PCR identified 12 major clonal types, respectively, among 198 isolates of *S. aureus* from bovine mastitis in Ireland and the USA. Three clonal types were identified among the 156 Irish isolates in the study and occurred in the following frequencies: RAPD type 5, 51%; RAPD type 7, 20%; RAPD type 4, 27%. These types were also identified among isolates from dairy cows in USA.

- Typing of multiple isolates from the same primary isolation plate suggests that infections caused by *S. aureus* are usually associated with a single strain.

- RAPD-PCR and ribotyping identified different molecular types of *S. aureus* isolates within individual herds.

- RAPD-PCR and ribotyping of American and Irish isolates identified subtypes within ET clonal types identified by multilocus enzyme electrophoresis (MLEE). This suggests that there is a greater genetic diversity of bovine *S. aureus* on a global scale than was previously thought.

- Pulsed field gel electrophoresis (PFGE) was used for fine structure analysis of the genetic diversity of bovine *S. aureus* and proved to be more discriminatory than the other methods utilised. It should prove useful in epidemiological analysis of outbreaks of bovine mastitis.

- Genomic characterisation of *S. aureus* from bovine mastitis revealed an association between clonal type and putative virulence factors. RAPD type 7 isolates produced toxic shock syndrome toxin (TSST) and Enterotoxin C whereas the other RAPD types did not.

- RAPD types 4 and 5 expressed clumping factor whereas RAPD type 7 isolates did not. Also RAPD type 7 isolates were sensitive to penicillin whereas only 17% of all other isolates were sensitive.

• These data demonstrate that it should be possible to predict to a high level of confidence some of the virulence factors expressed by an isolate of *S. aureus* on the basis of its genomic characterisation.

• Mouse mastitis model studies showed that RAPD type 7 isolates are more virulent than other types, demonstrating differences in the pathogenic ability of different clonal types of bovine *S. aureus* in Ireland.

• Development of a persistent active infection of the rabbit uterine horn which would mimic the subclinical infection of the bovine udder was not successful. During the first two weeks after infection, *S. aureus* induced an elevation in IgA and IgG levels. Thereafter there was a rapid decline to control levels, coincidental with the elimination of the organism from the chambers.

• The sequencing and characterisation of the genetic element encoding the genes responsible for toxic shock syndrome toxin (TSST-1) and staphylococcal Enterotoxin C (SEC) was completed and a 15.8 kbp variable genetic element was identified.

• This element fulfils the criteria for a pathogenicity island. It is likely that this element can move horizontally between strains and contributes to the virulence of the bacteria in causing bovine mastitis.

• In addition to tst and sec genes a gene with 40% identity to existing enterotoxins was identified on the element. This is currently being characterised to determine if it is a new Enterotoxin. There were at least 16 open reading frames of unknown function.

• Mutant *S. aureus* strains constructed by allele replacement of the genes encoding TSST-1 and SEC were analysed in an *in vitro* quantitative PCR assay. Activation of specific bovine Tcell subsets by TSST-1 and SEC was observed suggesting that *in vivo* these superantigens may be involved in host immunomodulation. This may

facilitate the persistence of *S. aureus* in the bovine udder.

- Preliminary studies show that the majority of Irish isolates contain genes for one or both of the newly identified enterotoxins SEG and SEI. It now appears that most bovine isolates have the capacity to produce enterotoxins suggesting that they may be clinically significant.

- Ninety-six of the Irish isolates and 39 of the USA isolates were tested for antibiotic sensitivity. Of the 96 Irish isolates examined, 55 (57.3%) were resistant to penicillin, compared with 16 of the 39 USA strains (41.0%). Among the Irish isolates, all 24 RAPD type 7 strains were sensitive to penicillin G, whereas 8 of 20 (40.0%) of the RAPD type 4 strains and 47 of 52 (90.4%) of the RAPD type 5 strains were resistant. All 39 American strains tested and all but three Irish isolates were sensitive to tetracycline. Only one American isolate and one Irish isolate were resistant to erythromycin. All the USA and Irish isolates were sensitive to neomycin, cephalothin and cloxacillin. Only two of the USA strains (5.1%) and none of the Irish isolates were resistant to novobiocin.

S. aureus is present in all dairy herds

INTRODUCTION

Studies on natural populations of *S. aureus* have identified considerable genetic heterogeneity. Thus, the effective control of mastitic infections caused by staphylococci may have to involve directed and rational strategies against clones of *S. aureus* commonly found in diseased udders.

Numerous methods have been utilised for discrimination and comparison of *S. aureus* isolates in epidemiological investigations of staphylococcal infections. More traditional biochemical and physiological typing methods such as bacteriophage typing, biotyping, antibiotic sensitivity testing, toxin and enzyme profiling, and plasmid screening have been superseded in the past decade by a plethora of molecular genetic procedures such as ribotyping, plasmid DNA restriction patterns, pulsed-field gel electrophoresis of macrorestriction DNA fragments, and random amplified polymorphic DNA (RAPD) polymerise chain reaction (PCR) analysis. These molecular techniques have proved useful for grouping isolates into convenient intraspecies subsets which has been of particular value in the monitoring of nosocomial spread of staphylococci and in the clonal analysis of methicillin-resistant staphylococci.

Multilocus enzyme electrophoresis (MLEE) analysis is another molecular technique which has been extensively employed in bacterial population genetics research. It indexes allelic variation in sets of randomly selected genes of the bacterial chromosome, thereby providing a means for estimating overall levels of single-locus and multilocus genotypic variation within a species.

In this study isolates from dairy herds in Ireland and the USA were typed using RAPD-PCR to analyse the degree of genetic diversity present among *S. aureus* isolates causing bovine mastitis.

Also representative isolates of Irish and American origin were typed using ribotyping, MLEE and PFGE to detect any further diversity and to create a fine-structure epidemiologic framework of bovine *S. aureus*. Background clinical information on *S. aureus* bovine isolates from herds in the south-west region of Ireland was available. It was proposed to identify if there are any clonal types, as identified by RAPD-PCR, which were more likely to be associated with a particular form of mastitic disease. Also, the degree of variation of isolates within a herd and within individual animals was to be determined. A limited number of repeat isolates were taken from cows with infected udders after antibiotic treatment and these were typed by RAPD-PCR.

The identification of virulence factors i.e., factors produced by an organism which contribute to its ability to cause disease is an important step in the elucidation of the disease process. In the context of the high frequency of infection, huge losses to the dairy economy and relative inefficacy of control measures, the development of a vaccine is of considerable interest. *S. aureus* produces a vast range of putative virulence factors which have been reported to be associated with disease in humans and animals including intramammary infection of cows.

In this study we examined a number of putative virulence factors which have been associated with *S. aureus* and to determine their frequency among bovine isolate populations and among the clonal types identified using RAPD-PCR.

Genes encoding superantigens may be associated with mobile genetic elements such as pathogenicity islands, phages and plasmids. Mobile genetic elements are likely to contribute to the horizontal transfer of virulence determinants between related species. Recently, the identification and characterisation of a pathogenicity island (SaPI1) of a human clinical isolate which contained the gene for TSST-1 (tst) and a gene with significant homology to the staphylococcal enterotoxins (ent-like), was reported. The authors reported that there was likely to be a family of such tst-elements which were related but quite distinct.

The mobility of SaPI1 was demonstrated by phage-assisted movement and site-specific integration into a recA- strain.

Pathogenicity islands are accessory genetic elements that range in size from 10 to 200 kbp, contain one or more genes associated with virulence, are bordered by directly repeated sequences, can be deleted *en bloc* and may have integrase-like genes. They are widely assumed to be mobile.

Preliminary studies in this laboratory showed that tst- and sec-specific probes appeared to hybridise to the same-size HindIII restriction fragment in Southern blot analysis suggesting that the genes may be contiguous on the genome. The current study identifies and characterises the associated genetic element, named bovine staphylococcal pathogenicity island (SaPIbovine).

Typing of bovine *S. aureus* isolates using RAPD PCR

In the present investigation, RAPD PCR identified 12 major clonal types accounting for over 98% of 198 isolates of *S. aureus* recovered from cows in Ireland and the USA (Fig. 1., Table 1). Among the 156 Irish isolates examined, there were 3 major clonal types as identified by RAPD-PCR. These three main RAPD types were identified throughout the country and were identified in the following frequencies: RAPD type 4, 27%; RAPD type 5, 51%; and RAPD type 7, 20%.

The isolates were taken from cows with different forms of mastitic disease, i.e. subclinical and clinical. There was no correlation between RAPD type and the clinical form of disease.

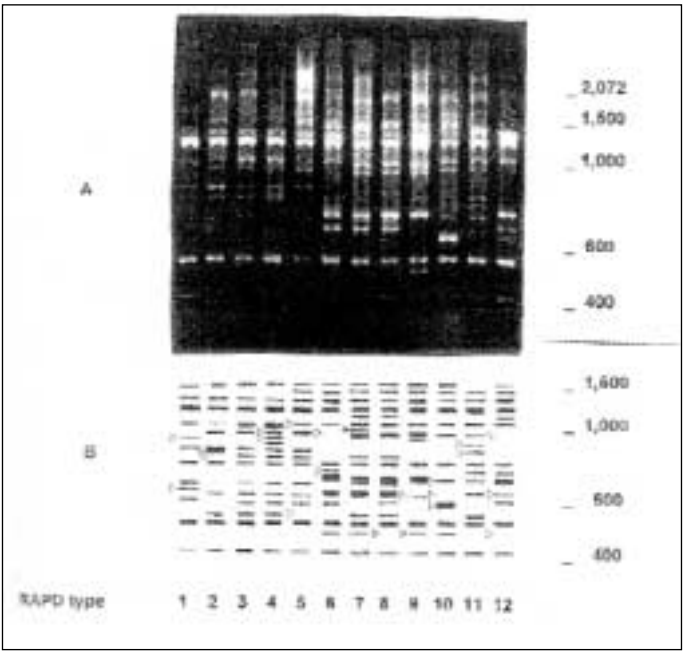


Figure 1 : Agarose gel electrophoresis of RAPD-PCR products representative of the 12 major fingerprint types among 198 isolates of *S. aureus* from cases of bovine mastitis, and corresponding schematic diagram.

Typing of multiple isolates from the same primary isolation plate

6 colonies from each of 6 primary isolation plates from a single cow were typed by RAPD-PCR. Only one fingerprint type was identified among the 36 isolates. Further studies of multiple isolates from primary isolation plates revealed identical RAPD types of isolates from a single plate. These data suggest that single strain infections are the predominant cause of bovine mastitis due to *S. aureus*.

Table 1 : RAPD types of *S. aureus* isolates from dairy herds in Ireland and US

RAPD type	Origin	No. of Isolates
1	USA	8
2	USA	4
3	USA	1
4	IRL	43
	USA	5
5	IRL	80
	USA	2
6	USA	2
	IRL	1
7	IRL	30
8	USA	6
9	USA	8
10	USA	3
	IRL	1
11	USA	2
12	USA	1
13	IRL	1

Typing of multiple isolates from the same herd

RAPD PCR identified different molecular types of *S. aureus* isolates within individual herds (Table 2). However, in most herds, a single clonal type appeared to predominate. This infers that bovine mastitis is of an infectious nature with *S. aureus* being passed from animal to animal *via* infected teat-cups or hands and suggests that infection of the udder by opportunistic environmental isolates of *S. aureus* is unusual.

Table 2 : RAPD types within individual herds

	No. of isolates of indicated RAPD types :		
Farm Location	5	7	4
Ballymacoda		1	
Ballyderown, Fermoy	19	6	2
Cappamore, Co. Limerick	2		
Curtin's, Fermoy	5	2	2
Cork	4	1	
Mitchelstown			17
Ennis, Co. Clare	6	5	
Mallow1	1		4
Mallow 2	5		
Moorepark	6	2	
Aghada, Co. Cork	3	2	
Tipperary	9	3	
Unknown (Dairygold Co-op) region	1	2	
Total	61	24	25

Comparison of RAPD PCR to other molecular typing techniques

RAPD PCR and ribotyping of American and Irish isolates identified subtypes within clonal types identified by multilocus enzyme electrophoresis (MLEE). RAPD and ribotyping proved to be more discriminatory than MLEE.

These results suggests that there is a greater genetic diversity of bovine *S. aureus* on a global scale than was previously thought. However, the same molecular types appeared to be associated with the disease in the U.S.A and Ireland. This evidence supports the hypothesis that there are only a small number of clones responsible for the majority of cases of bovine mastitis and that these clones have a wide geographic distribution.

Pulsed field gel electrophoresis (PFGE) was used for fine-structure analysis of the genetic diversity of bovine *S. aureus* and was found to be more discriminatory than the other methods. This method may prove useful in fine-structure epidemiological analyses of outbreaks of bovine mastitis.

Virulence factor analyses

Genomic characterisation of *S. aureus* from bovine mastitis revealed an association between clonal type and putative virulence factors. (Table 3) RAPD type 2 isolates produced toxic shock syndrome toxin (TSST-1) and Enterotoxin C whereas the other RAPD types did not. RAPD types 4 and 5 expressed clumping factor (fibrinogen-binding protein; fbp) whereas RAPD type 2 isolates did not. Also, all RAPD type 7 isolates were sensitive to penicillin and only 17% of all other isolates were sensitive. Staphylokinase (Sak) was produced by only four of the 144 isolates tested. Conversely, the g-haemolysin locus was harboured by all isolates analysed.

Table 3 : Production of putative virulence factors by bovine *S aureus* isolates

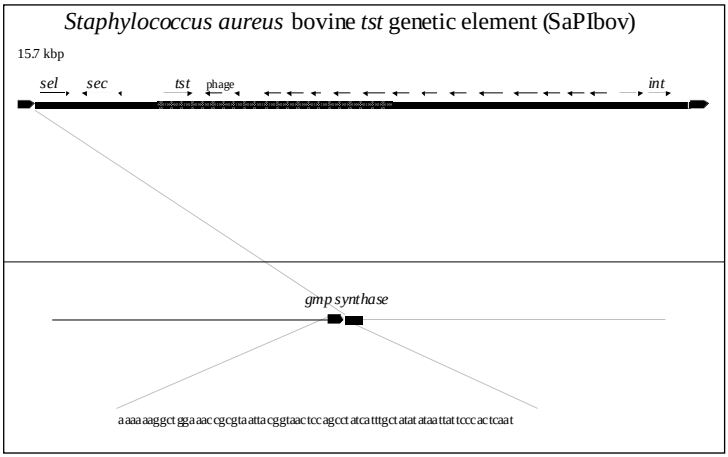
RAPD type	No. of Isolates	Fbp	SE C/D	TSST1	Sak
1	8 USA	6/8	0/8	0/8	2/8
2	4 USA	3/4	0/4	0/4	0/4
3	1 USA	1/1	0/1	0/1	0/1
4	5 USA	5/5	1/5	1/5	0/5
	26 IRL	25/26	1/26	1/26	1/26
5	2 USA	2/2	0/2	0/2	0/2
	52 IRL	52/52	0/52	0/52	0/52
6	2 USA	2/2	0/2	0/2	0/2
7	24 IRL	0/24	21/24	20/24	0/24
8	6 USA	5/6	0/6	0/6	0/6
9	8 USA	6/8	1/8	0/8	1/9
10	3 USA	0/3	0/3	0/3	0/3
11	2 USA	0/2	0/3	0/3	0/3
12	1 USA	0/1	1/1	0/1	0/1
TOTAL	144	107	25	22	4

These data demonstrate that it should be possible to predict to a high level of confidence some of the virulence factors expressed by an isolate on the basis of its genomic characterisation.

Characterisation of pathogenicity island (SaPI1)

The current study reports the identification and characterisation of a novel pathogenicity island (Fig. 2) of 15.7 kbp in length in a strain of *S. aureus* isolated from bovine mastitis. Multiple superantigens including TSST-1 and SEC and possibly a third, SEL, are encoded by the element. Currently, we are investigating, if this gene (sel) is expressed in *S. aureus* and if it has superantigens activity. It is possible that there are other putative virulence factors which are encoded among the 16 orfs of unknown function.

Figure 2



In-vitro superantigens functional analysis

Bovine Peripheral Blood Mononuclear Cells (BPBMCs) were incubated with supernatants of the tst and sec mutants and parent strain. Quantitative PCR of the BPBMCs using primers specific for the bovine T-cell Vbeta domains was then carried out.

Comparative analysis identified which T-cell subtypes were activated by the different superantigens.

It was found that TSST-1 stimulates T-cells with BoVbeta 18, 93 whereas, SEC activates BoVbeta 13.

This demonstrates how the superantigens are interfering with the host immune response *in vitro*.

Mouse mastitis model

Mouse mastitis model studies revealed that RAPD type 7 isolates are more virulent than other types, demonstrating differences in the pathogenic ability of different clonal types of bovine *S. aureus* in Ireland.

Rabbit infection model

The objective of this study was to use a bacterial diffusion chamber to set up a persistent active infection of *S. aureus* in the uterine horn of the rabbit. Based on the common system of mucosal immunity, this would mimic the subclinical situation in the mammary gland of the cow. The model would then be used to explore ways of enhancing the immune response to this important animal pathogen.

A total of 30 rabbits were used in the study. The strains of *S. aureus* were left in the chambers (in the uteri of the rabbits) for periods of 7, 14, 21, 28 and 42 days. At these predetermined time intervals, samples of secretion were collected from the uteri and these were assayed for IgA and IgG as indicators of a humoral immune response. During the first two weeks, all three strains induced an elevation in IgA and IgG levels. Thereafter, there was a rapid decline to control levels, coincidental with the elimination of the organism from the chambers. This level of persistence was considered to be insufficient to accomplish the objectives of this study. Further experiments could involve the use of progesterone (P4) to prolong the survival of *S. aureus* within the uterus.

CONCLUSION

- In Ireland only 3 clonal types of *S. aureus* were identified in association with intra-mammary infection. However, on a global scale there appears to be greater genetic diversity of bovine *S. aureus* isolates than was previously thought.

- Some clonal types as identified by molecular fingerprinting techniques were represented among isolates of American and Irish origin suggesting that they are highly-specialised strains with a broad geographic dissemination.

- Typing of multiple isolates from the same primary isolation plate suggests that single strain infections are the predominant cause of *S. aureus* bovine mastitis and RAPD-PCR and ribotyping identified different molecular types of *S. aureus* isolates within individual herds.

- Genomic characterisation of *S. aureus* from bovine mastitis revealed an association between clonal type and putative virulence factors such as TSST-1, Enterotoxin C and clumping factor. A correlation between RAPD type 7 isolates and sensitivity to penicillin was also revealed.

- Mouse mastitis model studies have shown that RAPD type 7 isolates are more virulent than other types, demonstrating differences in the pathogenic ability of different clonal types of bovine *S. aureus* in Ireland.

- These data demonstrate that it should be possible to predict to a high level of confidence some of the virulence factors expressed by an isolate and speculate on the potential virulence of a strain of *S. aureus* on the basis of its genomic characterisation.

- A pathogenicity island encoding multiple superantigens was identified and characterised. It is likely that this is a mobile element which contributes to the virulence of the organism in the bovine udder.

Overall we have shown that bovine mastitis is caused by a limited number of *S. aureus* clonal types in Ireland as identified by the typing methods utilised. There also appears to be a number of virulence factors associated with these clonal types which may be important in the pathogenesis of the disease. This will have important implications as a rational strategy for the prevention or treatment of the disease needs to be directed against clones that commonly cause disease. In addition, vaccine technology needs to incorporate factors which are of importance in pathogenesis and which are produced by the predominant strains associated with mastitis.

We have also characterised the first *S. aureus* bovine pathogenicity island and only the second one to be characterised in a gram-positive organism. This element may contribute to the virulence of bovine strains within the udder and/or allow persistence of bacteria contributing to the chronic nature of the disease.

PUBLICATIONS

Fitzgerald, J.R., Meaney, W.J., Hartigan, P.J. and Smyth, C.J. (1994). PCR-based random amplified polymorphic DNA fingerprinting of *Staphylococcus aureus* isolates from bovine mastitis. Proceedings of the Society of General Microbiology (Irish Branch) Symposium on Molecular Characterisation of Microbial Pathogens, Jordanstown, N. Ireland, September 1994, Abstract p5.

Fitzgerald, J.R., Meaney, W.J., Hartigan, P.J. and Smyth, C.J. (1994). Molecular Characterisation of *Staphylococcus aureus* isolates from bovine mastitis using RAPD PCR. Proceedings of the 31st Winter Scientific Meeting of the Association of Veterinary Teachers and Research Workers (Irish Region), Dublin, December 1994 Abstract, p4.

Fitzgerald, J.R., Meaney, W.J., Hartigan, P.J. and Smyth, C.J. (1995). Molecular Characterisation of *Staphylococcus aureus* isolates from bovine mastitis using RAPD-PCR. Abstract Irish Veterinary Journal Vol. 48. No. 9, p334.

Fitzgerald, J.R., Smyth, C.J., Meaney, W.J. and Hartigan, P.J. (1996). Ribotyping of *Staphylococcus aureus* recovered from cases of bovine mastitis in divergent geographic locations. International Dairy Federation Newsletter No 21, September 1996, p29.

Meaney, W.J. and Flynn, J. (1996). Antibiotic resistance of *Staphylococcus aureus* isolated from cases of bovine mastitis in Ireland. International Dairy Federation Newsletter No 21, September 1996, p28.

Fitzgerald, J.R., Meaney, W.J., Hartigan, P.J., Smyth, C.J., and Kapur, V. (1997). Fine-structure molecular epidemiological analysis of *Staphylococcus aureus* recovered from cows. Epidemiology and Infection 119 : 261-269.

Fitzgerald, J.R., Meaney, W.J., Hartigan, P.J. and Smyth, C.J. (1997). *Staphylococcus aureus* isolated from udders of cows in Ireland : genetic diversity identified by molecular fingerprinting techniques. Proceedings of Irish Grassland and Animal Production Association 23rd Meeting 3rd / 4th April 1997, pp 183-184

Meaney, W.J. and Flynn. J. (1997). Antibiotic Resistance Testing of *Staphylococcus aureus* isolated from cases of bovine mastitis. Proceedings of Irish Grassland and Animal Production Association 23rd Meeting 3rd / 4th April 1997 pp 183-184

Meaney, W.J. and Flynn. J. (1997). Antibiotic Resistance Testing of *Staphylococcus aureus* isolated from cases of bovine mastitis. Irish Journal of Agriculture and Food Research Vol. 36, No.1 : p113.

Fitzgerald, J.R. (1997). Population analysis of *Staphylococcus aureus* from bovine mastitis. Ph.D. Thesis. University of Dublin, Trinity College.

Fitzgerald, J.R., P. J. Hartigan, W. J. Meaney, and C. J. Smyth. (1999). Superantigens of *Staphylococcus aureus* from bovine mastitis. International mastitis meeting, Silver Springs hotel, Cork.

Fitzgerald, J.R., P. J. Hartigan, W. J. Meaney, and C. J. Smyth. 2000. Molecular population and virulence factor analysis of *Staphylococcus aureus* from bovine mastitis. J. Appl. Microbial, 88, 1028-1037.

Fitzgerald J.R., Monday S, Foster, T.J. Bohach G, Meaney, W.J., Hartigan P.J., Smyth C.J. 2000. A pathogenicity island from bovine *Staphylococcus aureus* encoding multiple superantigens. Accepted for publication, J. Bacteriology

NOTES