Original Article

Temporal expression of extracellular products of staphylococcus aureus in vivo mouse cage model

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Abstract

Objective: To test the hypothesis that Staphylococcus aureus genome has regulatory genes which coordinate the expression of extracellular products, and particular genes not expressed in vitro conditions may be turned on in a vivo environment.

Methods: The study was conducted at the Immunology and Infectious Disease Research Laboratory (IIDRL), Microbiology Department, Husein Ebrahim Jamal Research Institute (HEJ), Animal House, Karachi University, from July to December 2009. Micro pore Teflon cages using a mouse cage model were fixed into the subcutaneous tissue in Albino mice (BALB/c) on their dorsal surface. After 15 days, the holes closed down with healthy tissue. Three staphylococcal isolates from clinical samples confirmed by DNA sequencing of 16s ribosomal RNA were tested for expression of extracellular protein in vitro and were later injected into the cages. After the institution of infection, the fluid aspirated from the cages was analysed by Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE). This was done to test for possible induction of additional extracellular proteins in vivo.

Results: The appearance of enhanced extracellular products was observed in the fluid recovered from the cages of two mice on days 5 and 7 subsequent to the institution of infection, suggesting a turn-on of particular genes which were not expressed in vitro conditions.

Conclusions: In-vivo host and environmental signals contribute to the induction of genes for the production of extracellular proteins.

Keywords: Staphylococcus aureus, Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Mouse cage model (JPMA 62: 539; 2012).

Introduction

Staphylococci are pathogens that cause a range of infections from superficial to lethal, such as endocarditis, septicaemia, necrosis of fascia, and toxicosis, like Staphylococcus Scalded Skin Syndrome (SSSS) and Staphylococcal Toxic Shock Syndrome (TSST).[1] Staphylococcus possesses a cache of virulence factors that contribute in disease production; exoproteins including haemolysins, nucleases, proteases help in host cell destruction whereas cell wall bound components such as fibronectin protein and protein A, facilitate the institution of infection in host tissues and fight host immunity.[2] The exotoxins like enterotoxins, exfoliative toxins, and the TSST are super antigens that motivate significant inflammatory response and cause tissue damage.[3] Many of these virulence factors are under the command of a complex regulatory system that allows the synchronised expression of different genes.[4] The agr locus family genes of Staphylococcus aureus have a vital function in modifying virulence factors.[5] The mode by which these virulence factors are controlled and the effect of their expression on disease production is at the moment subject of research all over the world. A silkworm infection model using micro-organisms such as Staphylococcus aureus and Psuedomonas aeruginosa was created to learn the impact of genes controlling virulence factors.[6,7] A study by Chatellier on beta-haemolytic streptococci observed unpredictable expressions of Streptococcal pyrogenic exotoxins (Spes) in between clonal isolates from invasive infections.[8] Most note worthy was the fact that the expression of SpeA was not traceable in 40% of the isolates. The inability of Streptococcus pyogenes serotype M1 to produce discernable levels of exotoxin SpeA while additional organisms originating from the identical clone produce increased amounts of SpeA has remained unresolved. In an effort to tackle the subject whether SpeA gene induction can be turned on in vivo, a mouse cage model was developed by Kazmi et al.[9] They reported in their study that SpeA expression steadily occurred in vivo in clonal isolates expressing no traceable amount of SpeA production in vitro. In support of this theory that host factors participate in the regulation of virulence genes, the mouse cage model was adopted to study the expression of extracellular products of Staphylococcus aureus in vivo as compared to vitro conditions.

Material and Methods

Three isolates obtained from clinical samples like pus, urethral swab and blood were identified by standard microbiology techniques on the basis of colony characteristics; yellow to cream or occasionally white 1-2 mm in diametres, slightly raised colonies. After overnight incubation, some strains showed beta-haemolysis. Gram stain morphology, purple coloured grape-like clusters were observed. After tube coagulase test and mannitol fermentation test, these three isolates were further characterised by DNA sequencing of 16s ribosomal RNA.[10]

For the preparation of template DNA, overnight cultures in Brain Heart Infusion Broth (BHI) were centrifuged at 4,000 rpm for 10min. After the removal of supernatant, the pellet was suspended in 0.5ml of InstaGene Matrix (Bio-Rad, USA), incubated at 56°C for 30min and then heated at 100°C for 10min. The resultant supernatant was used for PCR. 1.0ml of template DNA in 20µl of PCR reaction solution along with universal primers. Next, 35 amplification cycles at 94°C for 45sec, 55°C for 60sec, and 72°C for 60sec were performed.

The purified PCR products of approximately 1,400 bp were sequenced by using universal primers. Sequencing were obtained by using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA) and deposited in GENBANK (a general sequence database).

Sequencing was performed on three Staphylococcus aureus isolates after DNA preparation and amplification by PCR. Sequencing of strain was carried out by using primers specific for16srRNA gene of Staphylococcus aureus. Each sequencing was performed by using Big Dye terminator cycle sequencing kit (applied BioSystems foster City, CA-USA).

PRIMERS: F-ccagcagccgcggtaatacg, R-taccagggtatctaatcc.

GENE: 16S ribosomal RNA.

For the purpose of Teflon Tissue Chamber Infection Model, six to eight weeks old BALB/c mice of female gender weighing about 22-25g each were obtained from the HEJ Animal Laboratory, Karachi University. All guidelines involving ethics of conduct with animals used in experiments were adhered to. The study was approved by the Dean of Sciences, Karachi University.

Sterilized Teflon FEP (Fisher, Suwannee, Georgia, USA) mouse cages 20 by 10 mm were taken, and 110 holes were made uniformly spaced at 1-mm diameter. Two bigger holes at each end were made to permit the penetration of a 27-gauge needle (Figure-1). It was constructed at the Department of Biomedical Instrumentation (University of Tennessee, Memphis). The autoclaved mouse-cages were fixed in to house individual mouse. The experiment was conducted in aseptic conditions inside a protective hood with a Bunsen flame. A minor cut in the skin to the depth of the subcutaneous connective tissue on the dorsal side of the mice was given, and the cage was inserted (Figure-2). The incisions were secured with 9-mm surgical clips, and povidone-iodine antiseptic was applied over the site of surgery to prevent infection. The mice were examined every day for any signs of infection. In the course of the experiment one mouse developed infection and shed the cage. In the other two mice, after 21 days the holes of the chamber closed

down with healthy connective tissue and the compartment was filled with a pale fluid. The fluid was checked for sterility by taking 1 ml of the fluid from the chamber and culturing on blood agar media for 24 hours at 37°C.

For the preparation of bacterial inoculums, three strains of Staphylococcus aureus confirmed by DNA sequencing of 16s ribosomal. RNA (Figure-3) were cultured overnight at 37°C in nutrient broth. The number of Colony Forming Unit (CFU) per ml was calculated and 1×10^3 to 5×10^5 CFU of the Staphylococcus strains being tested were inoculated in phosphate-buffered saline, into the mouse cages. To find out if the inoculums size influences the growth of the organisms within the confined cages, some isolates were injected in numbers ranging from 1×10^3 to 5×10^5 CFU. Despite the original inoculums size, a noteworthy rise in the amount of CFU per ml was apparent in the first week, but later it became stable when a size of 10^9 CFU/ml was achieved. When a small inoculums was used (i.e., 10^3 CFU/chamber) infection was not established. Infection was readily instituted in the mice injected with an inoculum of 10^5 CFU. Therefore, the mouse cages were inoculated with 100µl of Phosphate Buffer Saline (PBS) containing 10^5 CFU of the isolates. There was a marked rise in the total leucocyte count in the chambers by the end of the first week.

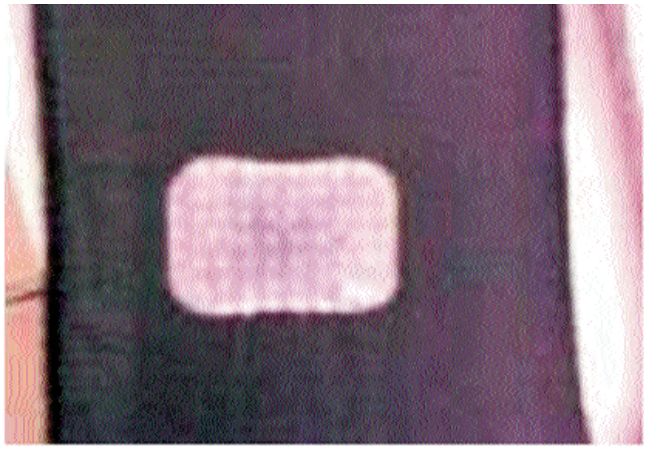

Figure-1: Tissue chamber used in localized infection model in mice. Teflon-FEP tissue chambers (20 by 10 mm) were perforated by 110 equally spaced 1-mm-diameter holes with a larger hole at one end to allow penetration of a 25-gauge needle.

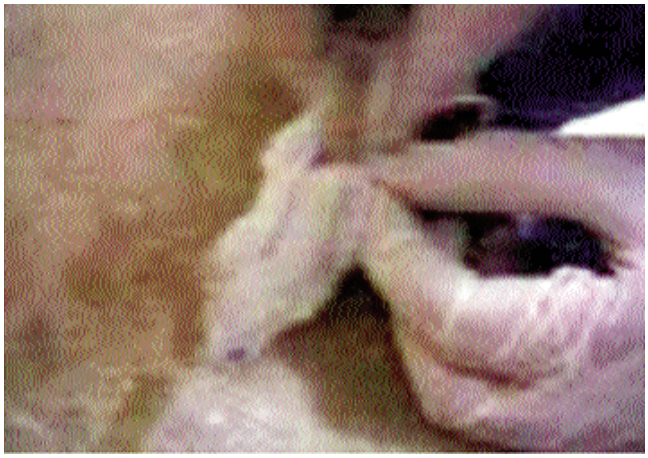

Figure-2: Teflon cage being inserted through a small incision in the subcutaneous connective tissue of the backsides of anaesthetized mice.

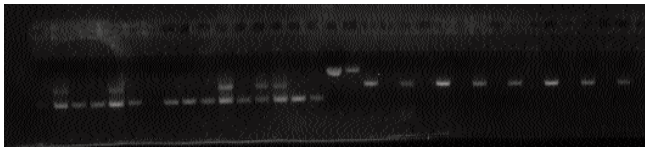

Figure-3: Gel electrophoresis of Staphylococcus.aureus isolates shown bands of 337bp. Lane M, 100bp DNA molecular mass Lane YT2; Lane YT3; Lane YT4.

The control animal whose chambers were inoculated with normal saline, showed no changes, including no white blood cell infiltration. After closing down of the cage with the connective tissue, while bacterial exo products and neutrophils could circulate in and out by diffusion and diapedesis, the bacteria remained confined to the chamber. This made it possible to take samples of S. aureus isolates from the chambers after the institution of infection in order to examine possible changes in the expression of extracellular products in host environment.

Isolates supernatants prepared before establishing the infection in vitro state was selected at day 1 and these were screened for extracellular proteins by sodium dodecyl sulfate gel electrophoresis.

The fluid from the chamber was aspirated 3 days post-inoculation. The liquid was recovered from two cages. In the third mouse, the incision became infected and the cage was shed. The fluid was centrifuged at 5000rpm for 30min to remove any mouse blood cells, bacteria and debris prior to analysis try sodium declecyl sulfate (SDS) gel.

For the purpose of exoprotein profiles, 12-hour cultures (2ml) were inoculated into a fresh broth (200ml) and centrifuged. The supernatants obtained were trichloroacetic acid which were precipitated and again centrifuged at 15k rpm for 30min. The pellets were washed twice with chilled ethanol and subjected to electrophoresis in a 12.5% polyacrylamide gel in accordance with the technique of Laemmli.[11]

The protein profile of the isolates was analysed by SDS-PAGE following the discontinuous buffer system of Laemmli. Samples were prepared for PAGE by combining in a ratio of one part of sample to one part of SDS buffer dye and boiling for 5min. A samples size of 20µl was applied to wells in a 5% acrylamide stacking gel over 12.5% acrylamide separating gel. Electrophoresis was performed using a Bio-Rad Mini-Protean 11 apparatus, with vertical slab gel 8.4 cm length into 8 cm width and of 1 mm thickness. An electric current of 100 volts was applied constantly till the tracking

dye reached the bottom of the gel over a time of 2 hours. Broad-range molecular weight markers (Bio-Rad) were also run for estimation of the bands of interest. After the completion of electrophoresis, the gels were removed and stained with Coomassie Brilliant Blue R-250. The gels were de-stained with 20% acetic acid and 10% methanol overnight. The gels were photographed and the relative molecular weights (Mr) of proteins bands obtained were determined by using statistic method of calculating standard linear regression curve of standard protein markers (MS Excel; version 2007).

The relationship between two variables, Rf value and log molecular weight of standard protein (Bio-Rad), was analysed. We constructed a standard curve between standard protein Rf value and the standard protein molecular weight and appllied linear regression curve to find the best predictable values using the MS-Excel ver-2007 software. RF values of standard protein were determined as the distance covered by the protein in the gel divided by the total length of the gel (8.4cm).

Results

The nucleotide sequences of three isolates were obtained from the gene bank. Their accession numbers were, GQ 214334, GQ 214332, and GQ 214333. The sequences of the three strains were posted at the gene bank (Figure-3).

Bacteria retrieved on days 5, 7 and 14 produced different extracellular products and maintained this phenotype for a period of three weeks. Isolates obtained on day 21 was the same as the original phenotypes, proposing a turn-off of the genes. The difference in response was noticed in several extracellular proteins.

Protein profile of Test 1 organisms showed almost identical results but with an increase in intensity in vivo as compared to vitro (Figure-4). More than 90% of bands were qualitatively and quantitatively matching with significant

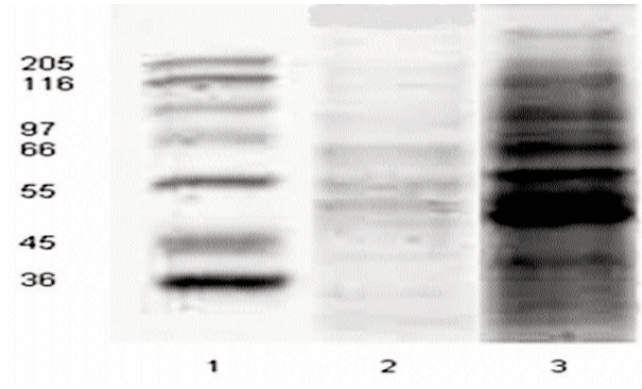

Figure-4: Test organism No.1 GQ21334 (Pus-swab) - SDS PAGE showing exaggerated bands in vivo mouse cage.
Lane-1: Protein Markers; Lane-2: Staphylococcus aureus - Expression of extracellular products in vitro before mouse inoculation (Day-1). Lane-3: Staphylococcus aureus - Expression of extracellular products in vivo after mouse inoculation (Day 7).

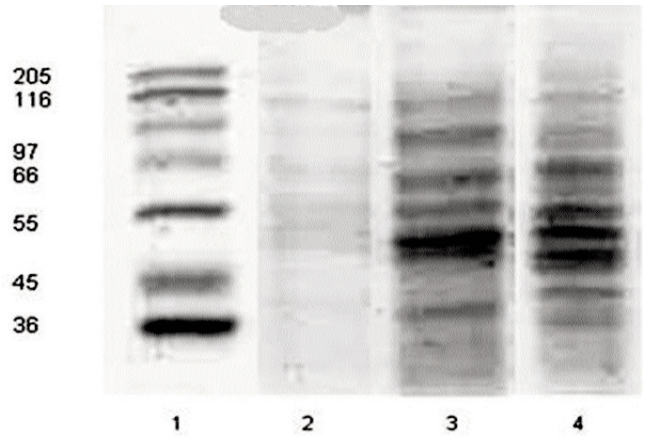

Figure-5: Test organism No. 2 GQ214332 (Urethral-swab) - SDS PAGE showing increased number of bands in vivo mouse cage.
Lane-1: Protein Markers; Lane-2: Staphylococcus aureus - Expression of extracellular products in vitro before mouse inculation (Day 1). Lane-3: Staphylococcus aureus - Expression of extracellular products in vivo after mouse inculation (Day 5). Lane-4: Staphylococcus aureus - Expression of extracellular products in vivo after mouse inculation (Day 7).

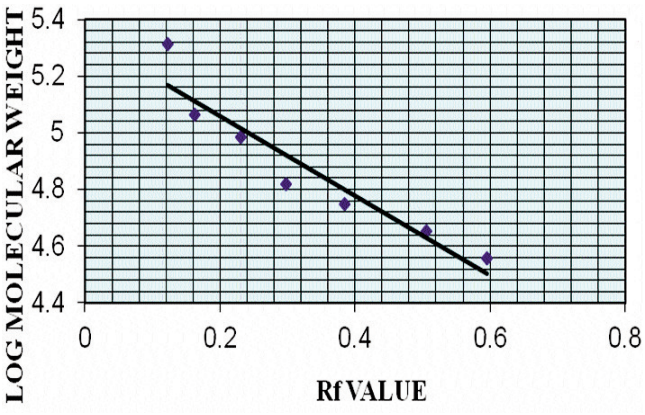

Figure-6: Standard Linear Regression Curve between Rf-Values and Standard Protein Marker Molecular Weight.

increase in concentration in the bands on day 7 (Lane 3), obtained by SDS after in vivo inoculation. Standard molecular weight markers of a wide range (Bio-Rad) were also run (Lane1).

The protein profile of Test 2 organisms showed a qualitative and quantitative difference in the pattern of bands in vivo as compared to the parental strand in vitro (Lane 2) (Figure-5). The variation in response was noticeable in a number of products on day 5 and 7 (Lane 3 and 4). Results indicated that the in vivo exposure to host environment triggered the expression of relevant genes which coded for the additional bands seen; these bands were absent in the clinical isolates extra-cellular products grown in vitro (Lane 1 of Test organism 1 and 2). Two significant protein bands corresponding to 140 kDa and 36 kDa ECPs were identified.

Table: Relative molecular weights (Mr) of staphylococcus aureus extra cellular products in vitro and vivo as calculated by linear regression curve.

Test - 1		Test - 2		
In vitro (Day 1)	In Vivo (Day 7)	In vitro (Day 1)	In Vivo (Day 5)	In Vivo (Day 7)
Mr	Mr	Mr	Mr	Mr
186	174	135	186	195
162	141	123	170	182
151	123	102	151	170
135	102	89	141	140
126	85	81	132	138
112	81	66	120	123
102	65	62	110	110
80	58	59	102	105
62	50	50	98	100
54	38	45	87	89
45	36	37	76	80
39	29		73	76
32	27		65	62
26	26		55	59
	7		51	55
			44	50
			39	43
			35	36
			29	37
			26	32
			25	29
				27
				13
				2

An attempt to categorise any of the protein bands was not made, but it can be assumed that, as different extracellular proteins, they are easily recognised by the immune system of the host. The approximate molecular weights of extracellular products in vitro and vivo in our results were calculated by the linear regression curve (Figure-6, Table)

Discussion

Staphylococcus aureus is an effective pathogen with the capacity to colonise and infect hospitalised patients with or without immune deficiencies as well as immunologically healthy people in the community[10] The traditional characterisation of bacteria on the basis of phenotype is generally not as correct as identification on the genomic level. As such, the comparison of the 16S rRNA has emerged as the preferred tool for bacterial characterization.[11,12] Accordingly, the strains utilised in our study were first characterised by genotype sequencing of 16SrRNA. Globally, many studies are being carried out in scrutinising mechanisms controlling transcription and/or post translational adaptations of key staphylococcal virulence factors, but little knowledge exists regarding their regulation.[8,9,11,13] Staphylococcus aureus has a diverse range of cell-associated and extracellular components that take part in disease production. The toxins, enterotoxins, exfoliative toxins, staphylococcal toxic shock syndrome (STSS) toxin are considered to be important virulence factors because of their potent super antigen properties.[2,3] There is considerable knowledge regarding the role of these factors in the development of infection, but little is known about the part played by the host. Worldwide, regulatory genes which organise the expression of various staphylococcal genes have been identified. The most broadly studied gene agr induces the expression of exoproteins through a bacterial-density-sensing mechanism.[13,14] Bound proteins are mainly synthesised during the exponential growth stage and the extracellular proteins are synthesised during the stationary stage. This sequential expression of genes may have a clinical significance. Different phases of staphylococcal infection may require different sections of virulence determinants. During the primary stages of infection-bound proteins that attach to extracellular-matrix molecules favour colonization, whereas in the next stage, synthesis of exo-products favour its spread. This hypothesis has been supported by animal studies showing that regulatory genes can be switched on and off according to environmental conditions.[15] In our study, results indicated that the in vivo exposure of the identified Staphylococcus aureus strains triggered the expression of some particular genes which coded for the additional bands seen; these bands were absent on SDS-PAGE performed on extracellular products when the same isolates were grown in vitro prior to inoculation. Two significant SDS-PAGE protein bands corresponding to 140 kDa and 36 kDa ECPs were identified The role played by 140kDa protein in virulence is that it is the root cause of

adhesion to polymer surfaces, leading to colonization, and so has significance in infections associated with foreign devices. The 34-36kDa protein has a significant role is in cytotoxic destruction of host cells.[16,17] This data supports the hypothesis and unlocks the doors for further research into the fundamental mechanism accountable for this observation. To observe the in vivo expression over a considerable period of time, it was essential to develop a non-lethal infection model. The mouse cage model of Kazmi et al[9] was adopted to explore the effect of host pathogen interaction on the induction of bacterial virulence genes. The micropore Teflon mouse cage was an adaptation of the steel cage model that was constructed by Nordstrand et al.[18] TheTeflon micropore model allows sequential sampling at different times after the institution of infection from the same mouse. These features diminish the chances of unpredictability if different animals were employed. Thus, the Teflon mouse cage combines the favourable characteristics of the air pouch model and the originality of the steel chamber model.[18,19] A bacterial setting which works in the in vivo unreceptive surroundings may have activated genes for the expression of extracellular products.

In Staphylococcus aureus infections, the accessory-gene-regulator (agr) quorum-sensing system plays an important role in virulence and is environment dependent.[20] It was also, therefore, taken into consideration that the induction of the extracellular proteins in the cage might be due to inter-bacterial contact signal as a result of growth stage due to inability of the bacteria to leave the confinement of the cage.[21]

A study carried out in Japan confirmed that the cvfB gene actively modulates induction of the agr locus, a virulence regulator in S.aureus.[21,22] The observations obtained from analysis using the silkworm infection model and the mouse infection model also concluded that the cvfB gene contributes to virulence via the agr-independent pathway. The concurrent regulation of two pathways, agr-dependent and agr-independent, by the cvfB gene is supposed to be essential for the regulation of virulence in S.aureus. The sarA gene also plays a part in the expression of virulence genes via agr-dependent and agr-independent pathways.[23] The existence of unknown pathways that regulates the induction of the agr locus and other modulatory transcription-factors according to environmental signals have yet to be identified.[24,25] These genes are supposed to interact with each other and to contribute to the regulation of virulence factors, although the whole representation of the regulatory system pathogenic gene expression remains to be identified.

Our results demonstrated a much stronger signal for certain proteins in vivo as compared to the parental strain. This suggests that an environmental signal in vivo has caused certain genes to be activated, which were not expressed in vitro and, therefore, influenced the severity of disease progression in the host. No attempt was made in the study to categorise any of the protein bands, but it can be presumed that, as extracellular proteins, they are easily recognised by the immune system after release from the organism.

Conclusion

The study suggests that in vivo host and/or environmental signals induced certain genes for the expression of certain extracellular products which were not expressed previously in vitro environment, and these may have an impact on the progress of the disease. Further studies need to scrutinise the in vivo signals essential for turning the genes on and off. Future testing may provide a molecular appreciation of immunity against Staphylococcus aureus and make it possible for the development of a vaccine that can protect humans at high risk of invasive Staphylococcus aureus infection.

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