Highly epidemic strains of methicillin-resistant *Staphylococcus aureus* (MRSA) do not differ from other MRSA or methicillin-sensitive strains in capsule formation, Protein A content or adherence to HEp-2 cells

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Abstract During the 1990s, two strains of epidemic methicillin-resistant *Staphylococcus aureus*, designated 'phage types EMRSA-15 and EMRSA-16, have emerged as significant hospital pathogens. They have resisted standard methods of control and spread widely amongst in the UK, often becoming endemic, while the incidence of other epidemic types of MRSA has either declined or not changed. This suggests that EMRSA-15 and EMRSA-16 possess special properties that favour their dissemination and survival. In order to investigate this hypothesis, a study was undertaken that examined methicillin-sensitive and methicillin-resistant strains of *Staphylococcus aureus,* including EMRSA types 1, 2, 3, 15 and 16, for capsule formation, the amount of bound protein A produced, and quantitative adherence to the human continuous epithelial cell line HEp-2. Although all these properties varied amongst the strains examined, there was no relationship between any of them and methicillin resistance or epidemic type, and, incidentally, no relationship between cell-wall bound protein A content and adherence.

Introduction

In recent years, methicillin- and multiply antibiotic-resistant strains of *Staphylococcus aureus* (MRSA) have caused outbreaks of hospital infection throughout the world. Some MRSA strains have particular abilities to spread in hospitals (and sometimes into the community) and have been called "epidemic methicillin-resistant *Staphylococcus aureus*" (EMRSA) [1-3]. New strains designated EMRSA-15 and EMRSA-16 appeared for the first time in the North and Midlands of England in the early 1990s, and since then have spread throughout the UK [4-7]. The dramatic spread of these two strains over the last six years has changed the UK from a country where MRSA was only a sporadic problem to one where it is now endemic, necessitating a major revision of national guidelines for control [8]. EMRSA-3, which is now the third most common epidemic strain, has changed little in its incidence during the 1990s, suggesting that the emergence of the highly epidemic EMRSA-15 and EMRSA-16 strains is the result of organism factors rather than changes in medical practice or patient casemix. One possible organism factor involved in colonisation and spread might be an enhanced ability of MRSA to colonise human mucosa.

The factors involved in staphylococcal colonisation of nasal epithelium are poorly understood. The organisms must adhere or bind to the cells (or to nasal mucin [9]) and resist the normal epithelial defensive and cleansing mechanisms [10]. A number of staphylococcal adherence factors related to invasion and infection have been identified, but their role in colonisation of intact epithelium is unclear. These factors

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include those expressed by human cells, such as vitronectin, fibronectin, fibrinogen, elastin, collagen and heparan sulphate [11-15], and those expressed by the bacterium, including lipoteichoic acid, techoic acid, protein A [16-18] and capsular polysaccharides [19].

Several workers have investigated the adherence of staphylococci to human nasal epithelial cells or to cultured cell lines. Duckworth & Jordens [20] compared the ability EMRSA-1 and other MRSA and MSSA to adhere to HEp-2 cells, and Ward [21] measured adherence of MRSA and MSSA to nasal mucosa, but neither study found any significant differences. Poston et al. [22] investigated the adherence of EMRSA-1 and other MRSA strains to HEp-2, Vero and mesothelial cells and showed no significant differences from results previously reported with MSSA strains [23]. However, they noted that deletion of the *mec* gene from one of the EMRSA-1 strains was associated with co-deletion of *agr* and *spa*, reduced production of protein A and reduced adherence.

In order to determine whether highly epidemic strains of EMRSA differ in their abilities to adhere to human epithelial cells in vitro, we have measured the adherence of a variety of EMRSA and MSSA to cultured HEp-2 cells, using ^{3}H-thymidine labelled bacteria in a quantitative assay. We also performed parallel microscopic studies to assess the patterns of adherence and the presence or absence of a capsule, and we measured cell wall-bound protein A concentrations.

Materials and Methods

Bacterial Strains. We used 13 clinical isolates of MRSA collected in the late 1980s and early 1990s during outbreaks at Guy's and St. Thomas' hospitals in London. They were selected so as to include examples of the common British epidemic strains EMRSA-1, EMRSA-2, EMRSA-3, EMRSA-15 and EMRSA-16. EMRSA-1 and EMRSA-16 are highly epidemic and several isolates of these from different years were examined; EMRSA-15 is also highly epidemic, but only one isolate of this type was available. Organisms were identified by standard methods, including the determination of tube-coagulase, and DNAase-production. Methicillin-susceptibility was identified by the formation of a 5mm diameter clear zone around a 5μg methicillin disc on blood agar plates incubated for 24h at 30^{o}C; methicillin-resistance strains showed no inhibition zone. The MRSA strains were kindly ‘phage typed by the Staphylococcal Reference Laboratory of the CPHL (Table 1). *Staphylococcus aureus* control strains were Wood 46, a methicillin-sensitive strain which is known to have relatively low adherence to HEp-2 cells [23]; Cowan I, another methicillin-sensitive strain known to have relatively high adherence to HEp-2 cells and which produces relatively large amounts of protein A [17], and the 'Oxford staphylococcus', NCTC 6571, another methicillin-sensitive strain.

Human Cell Line. This was human larynx carcinoma, continuous epithelial cell line HEp-2, (Imperial Laboratories, UK), that has been used previously in studies of staphylococcal adherence in our laboratory [23].

Chemicals, Media, Reagents and Plastic Ware. [methyl-^{3}H] thymidine was obtained from Amersham International, UK; Hisafe Optiphase scintillation cocktail from Wallac, UK; brain heart infusion (BHI), Mueller Hinton broth (MHB), Lauria Bertani broth (LB) and tryptose soy broth (TSB) from Unipath, UK; Trypan blue and Giemsa stains and recombinant protein A from Sigma-Aldrich, UK; and the following tissue culture materials were from Imperial Laboratories, UK: Earls Balanced Salt (EBS) solution, Dulbecco's phosphate-buffered saline (PBS), foetal bovine serum (FBS), Eagle's minimum essential medium with Earl's salt (EMEM), L-glutamine, penicillin and streptomycin, and 0.25 % trypsin with 0.02% EDTA in PBS without Ca^{2+} and Mg^{2+} . Tissue culture plastic ware and 50ml polyethylene centrifuge tubes were from Corning Costar Corporation, Cambridge, USA; Immulon 2 microtiter plates from Dynex Technologies, UK;

peroxidase conjugated antibody to goat IgG, peroxidase substrate system 3,3',5,5'-tetramethylbenzidine (TMB) and wash solution containing 0.002M imidazole and 0.02% Tween 20 in buffered saline from Kirkegaard & Perry Laboratories, USA; and India Ink from Windsor & Newton, UK.

Radiolabelling of Bacteria. Bacterial strains were radiolabelled as follows: five or six colonies from a fresh, 16 h culture on blood agar were picked by touching with a sterile wire loop and transferred to 2.5 ml TSB in a sterile 50 ml Corning tube. The bacterial cells were resuspended by vortexing and incubated overnight at 37^{o}C in the presence of 0.925 Mega becquerel ^{3}H-thymidine, in an orbital shaker at 175 rpm. The radiolabelled bacterial cells were harvested by centrifugation and washed twice in 20 ml of PBS. The washed bacterial cells were centrifuged, the pellet resuspended in EBS to an optical density (OD) of 0.4 OD_{540} to produce about 10^8 cells/ml [23].

Determination of Specific Activity of ^{3}H-labelled Bacteria. For each strain, two 100 µl aliquotes of the ^{3}H-labelled bacterial suspension was transferred to a 1.5 ml Eppendorf tube containing 1 ml scintillant, and the radioactivity measured in a Micro Beta Trilux liquid scintillation and luminescence counter (Wallac).

Cell Culture and Adherence Assay. 10^5 cells/ml of HEp-2 cells suspended in EMEM were incubated for 48 h at 37^{o}C in 24 well tissue culture plates to form confluent monolayers at the bottom of each well. The confluency and integrity of each monolayer was confirmed microscopically. Spent media was gently aspirated and the monolayer washed in 1 ml EBS. 0.5 ml of the ^{3}H-labelled bacterial suspension was added to each well and the plate incubated at 37^{o}C in a humid 5% CO_2 incubator for 90 min. After incubation, the monolayer was washed twice with 1 ml fresh EBS and then lysed by adding 250 µl of pre-warmed trypsin/EDTA solution and incubating at 37^{o}C for 15 min. Following complete detachment and solubilisation of the monolayer, 1 ml of scintillant was added and the mixture mixed in an orbital shaker until a clear solution was obtained. The radioactivity of each well was then counted in the Microbeta Trilux counter by inserting cross-talk elimination tubes into each well and using the Costar Software program with insert protocol (Wallac). The percentage of adherence was calculated as follows:

$$\%\,of\ adherence = \frac{mean\,cpm\,of\ the\,lysed\ monolayer\ with\,adherent\ bacteria \times 100}{mean\,cpm\,of\ the\,total\ bacterial\ suspension\,added}\,.$$

For each strain, eight replicates made from separate subcultures were tested and the median percentage adherence was used in comparisons.

Pairwise Comparison With negative Control Strain Wood 46. Figure 1 gives box-and-whisker plots associated with the adherence data in Table 1, and the 16 strains given in the table were compared using the Kruskal-Wallis method. This gave a P value of 3.6×10^{-7}, indicating that at least one of the strains was displaying adherence values significantly different from the rest. In order to identify which pairs of strains were significantly different, we used a multiple-comparison method [24]. This approach takes account of all the data present without compounding Type I errors. For this purpose, we used the non-parametric Dwass method [25], as described by Sokal and Rohlf [26]. According to the Dwass method, a pair of samples is significantly different at the α level if the Mann-Whitney U statistic for the pair is greater than or equal to the critical value $U_{\alpha[g,n]}$. This critical value is dependent on the number of groups g and on the number of values n in each group. With n=8, the largest possible value for U is 64, but $U_{0.05[16,8]}$=64.3, which makes $U<U_{0.05[16,8]}$ for all possible U. Therefore, in order to increase the power of the test, we omitted strains Wood 46 and 229.8 from this analysis on the grounds that their distribution was somewhat similar to that for strain GH177. This gave $U_{0.05[14,8]}$=63.9; consequently, two strains are significantly different at the 5% level if their box-and-whisker plots shown in Figure 1 do not overlap.

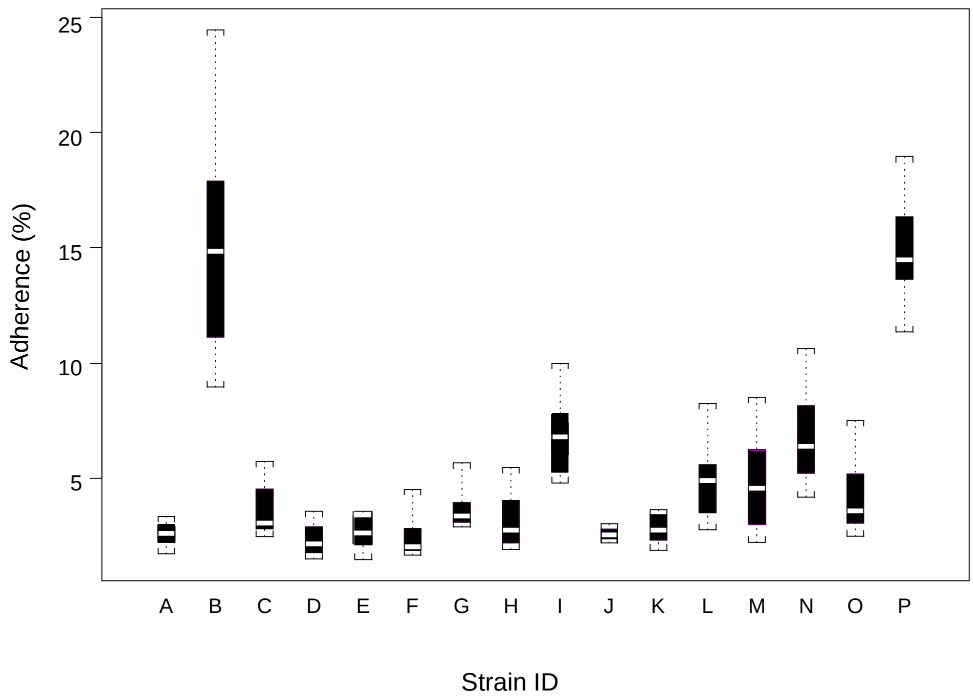

Figure 1 Box-and-whisker plots for replicate adherence experiments of *Staphylococcus aureus* isolates A-P shown in Table 1. See text for explanation

Spearman-Rank Correlation Analysis. A scatter plot of median percent adherence versus protein A concentration is given in Figure 2. The amount of correlation between median percent adherence and protein A concentration was assessed by the Spearman rank test.

Determination of Pattern of Adherence by Light Microscopy. HEp-2 cell monolayers were grown on 13 mm diameter, poly-L-lycine treated glass coverslips by placing the coverslips in the wells of tissue culture plates, seeding them with 10^5 HEp-2 cells and incubating to confluency as described above. A bacterial adherence assay was performed as before, but instead of lysing the cells with trypsin, the coverslips were removed, dehydrated with alcohol and stained with either Giemsa or crystal violet. The stained coverslips with their epithelial monolayers and adherent bacteria were examined by light microscopy at x400 and x1000 magnifications.

Examination of Capsules by Light Microscopy. According to Wilkinson's [27] practical definition, a capsule is a covering layer outside the cell wall, demonstrable by light microscopy and having a thickness greater than 200 nm and a definite external surface. We investigated all the bacterial strains used in this study for the existence of macrocapsules by the method of Duguid [28]. Fresh colonies grown on blood agar were suspended in PBS to a density of about 2×10^8 cfu/ml, and mixed with India Ink in ratio of 2:1. This was examined under glass coverslips using phase contrast microscopy at x600 and x1500 magnifications.

Protein A Quantitation. Cell bound protein A content of the isolates was determined by a modification of the enzyme immunosorbent assay (EIA) of Takeuchi et al. [29, 30]. Briefly, 100 µl of a formalin-treated bacterial suspension containing about 2 -3 x 10^8 cfu/ml was coated onto wells in 96 well microtiter plates by incubating at 37^{o}C for 16h. After washing, 100 µl of peroxidase-conjugated antibody to goat IgG was added at a 1:2000 dilution and incubated for 1 h at room temperature. (This antibody binds with protein A). This was followed by thorough washing of the wells, the addition of 100 µl TMB substrate solution, a short incubation until the development of a blue colour and then stopping the reaction by the addition of 100 µl 1M phosphoric acid. The optical density of the resulting yellow-coloured reaction mixture was measured at 450 nm using a Multiskan MCC 340 MK II EIA plate reader (Flow Laboratories, UK). Appropriate concentrations of recombinant protein A were used as standards in each plate and the quantity of protein A in each strain was calculated from the standard curve.

Results

Microscopic Assessment of Adherence. At 1000x magnification, the microscopic field typically covers an area of 0.01mm^2 and encompasses a minimum of 100 epithelial cells. In such fields, regardless of the staphylococcal strain investigated, we observed a great variation in the adherence of bacteria to HEp-2 cells, varying from a few tens to many hundreds of bacteria per cell. Furthermore, the distribution of the adherent bacteria was not uniform across each Hep-2 cell, but was concentrated close to the intercellular junctions. Furthermore, even a known low-adhering strain such as Wood 46 usually showed a large number of bacteria adhering to individual HEp-2 cells at some location in the field. Thus, it was not possible to make a quantitative assessment of adherence by microscopic analysis alone.

Specific Activity of ^{3}H-thymidine Labelled Strains of Staphylococcus aureus. There were considerable differences in the amount of ^{3}H-thymidine incorporation amongst the various strains studied, and it was also found that this varied with the culture medium used. However, with TSB the amount of incorporation was consistent for a particular strain, and this medium was therefore used throughout the study.

Adherence of Different Staphylococcus aureus Strains. The eight replicate percentage adherence results and the median values for each strain are shown in Table 1. Median adherence values ranged from 2.02% to 14.85%. The control strains Wood 46 (a known low-adherer) and Cowan I (a high-adherer) had values of 2.61 and 14.85 respectively. Figure 1 shows the box-and-whisker plots for the data in Table 1.

Statistical analysis

Using this analysis, Strains Cowan I (MSSA, mean adhesion 14.85%), GH13 (EMRSA-1, 14.48%), 114.1 (EMRSA-16, 6.78%), and 99.6 (EMRSA-1, 6.39%) had significantly increased adherence compared with the ‘negative’ control strain Wood 46 (MSSA, 2.61%). Thus, amongst the 16 strains tested, one MSSA strain had high adherence (Cowan I), as did two out of four EMRSA-1 strains and one of five EMRSA-16 strains. The single strains of EMRSA-2, EMRSA-3, EMRSA-15, non-typable MRSA, and the other MSSA strain (the Oxford staphylococcus) were not significantly different from the Wood 46 ‘negative’ control.

Relationship Between Adherence and Cell-Wall Bound Protein A Content

Cell-wall bound protein A content varied amongst the 16 strains from 2170 ng per 10^8 bacterial cells for the high-adherer control MSSA strain Cowan I, to 170 ng per 10^8 cells for the low-adherer control MSSA strain Wood 46. However, amongst the remaining strains, there was no association between protein A content and adherence, as shown by the scatter plot in Figure 2 and the Spearman rank correlation coefficient of 0.158 (P >0.2). Furthermore, there was no association between protein A production and methicillin resistance.

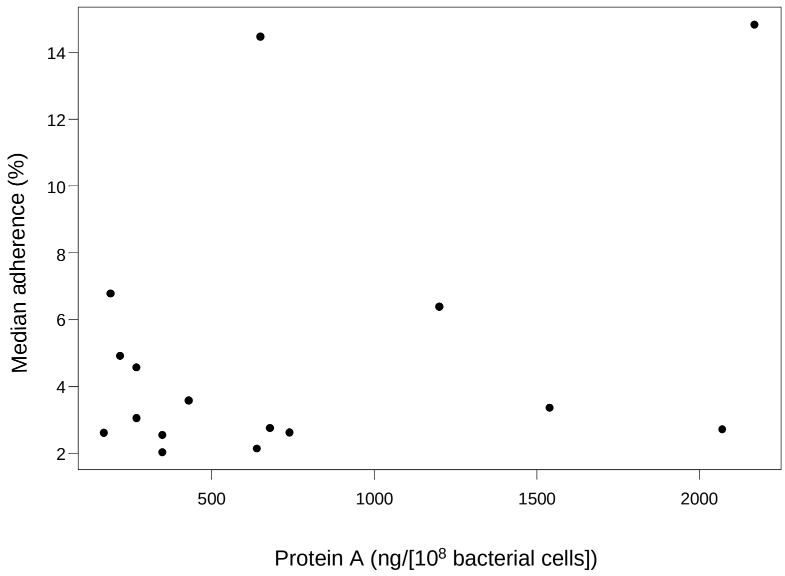

Figure 2 Scatter plot of mean percentage adherence versus cell-wall bound protein A content for the *Staphylococcus aureus* isolates tested.

Capsules and Adherence. No macrocapsule was detected in any of the strains examined except for 114.1 (EMRSA-16) and 86900 (EMRSA-l), which had thin capsular structures. None of the strains produced a mucoid colonial morphology on blood agar.

Discussion

Although others have used microscopic visual analysis for quantifying bacterial adherence to mammalian cells [9, 31, 32], we found this an unsatisfactory method. Accurate quantitation was difficult because adherence varied between individual cells and between different microscopic fields, a phenomenon that has been noted by others [23]. The radiometric system developed by Wyatt et al., [23] is more objective and reproducible and preferred for these studies. We used a modification of the Wyatt method in which the final specific activity of the cell lysate was measured directly in the tissue culture well itself. This is a simpler procedure and less prone to errors than having to remove lysates from the wells for radioanalysis. The problem of labelled bacteria adhering to the side walls of the wells was solved by using cross-talk eliminator inserts.

The expression of adhesion molecules in *Staphylococcus aureus* may be dependent on the growth phase and the composition of the culture medium [33, 34]. This is another source of variation that must be taken into account in adhesion experiments. We used TSB medium and stationary phase cells in this study.

Different mammalian cells differ in their expression of various staphylococcal adherence receptors, and therefore the cell line used in adherence studies will affect the results. Fibronectin is one of the many binding sites (receptors) for *Staphylococcus aureus* [11-13, 16]. The transformed cell line HEp-2, lacks fibronectin [35-37]. However, Wyatt et al., [23] reported that the addition of fibrinectin to the adherence assays did not significantly reduce the adherence of Cowan I to HEp-2 monolayers, and HEp-2 has been used in other studies of *Staphylococcus aureus* adhesion [20, 22]. For these reasons we believe HEp-2 is a suitable cell line for these studies.

With this methodology, the measurement of bacterial adherence was quantitatively reproducible, as shown by the inter-quartile range values (IQR) for eight replicates for each strain (Table 1). The mean adherence values for the control low-adhering and high adhering MSSA strains Wood 46 and Cowan I were 2.61% and 14.85% respectively, which are in accordance with the expected results. In addition to the MSSA Cowan I control strain, two out of the four EMRSA-1 strains tested had significantly increased adherence compared with Wood 46, as did one of the five EMRSA-16 strains. Adherence values for the single strains of EMRSA-2, EMRSA-3, EMRSA-15, non-typable MRSA, and the Oxford staphylococcus MSSA control strain were not significantly different from that of Wood 46. Thus in this study of 16 strains of *Staphylococcus aureus* there was no relationship between adherence to HEp-2 cells and methicillin-resistance, 'phage type or apparent clinical epidemicity. Furthermore, adherence values varied significantly amongst different isolates of EMRSA-1 and EMRSA-16. Thus, adherence in this assay is affected by factors other than methicillin resistance and epidemic type.

One factor that has been associated with adherence of staphylococci is protein A expression. Protein A is an extracellularly secreted and cell-wall associated protein of *Staphylococcus aureus* which interacts with immunoglobulin molecules in a pseudo-immune Fc reaction [38]. Various studies have suggested that Protein A expression is associated with increased adhesion to human cells [17, 22, 39], reduced susceptibility to opsonisation [18] and increased virulence [40]. The highly adherent *Staphylococcus aureus* strain Cowan I is known to produce higher amounts of protein A than the low-adhering Wood 46 [22] and is estimated to contain 80,000 IgG binding sites per organism [41]. Freney et al. [42] found that epidemic strains of MRSA had increased Protein A gene polymorphism, but van Belkum et al., [43] found no relation between gene polymorphism or protein A expression and colonisation of nasal epithelium. Poston et al. [22] demonstrated that *mec*$^-$ *agr*$^-$ *and spa*$^-$ mutants of a *spa*$^+$ MRSA strain had less protein A and adhered less well to cell lines. However, in another strain, a *mec*$^-$ mutant retained the parent levels of protein A while also showing decreased adherence. Finally, van Wamel et al., [44] found a significant difference between the ability of epidemic and sporadic strains of MRSA to bind vitronectin and Fc fragments of immunoglobulin G.

Protein A content has not been previously measured in human, clinical epidemic strains of MRSA. In the present study we measured cell wall-bound protein A quantitatively. As expected, the high- and low-adhering control strains had high and low levels of protein A respectively. However, there was no association between cell-wall bound protein A content and either adherence or methicillin resistance amongst the other *Staphylococcus aureus* strains tested.

Approximately 90% of *Staphylococcus aureus* isolates are said to produce capsular polysaccharides [45]. Encapsulated strains are more virulent in mice models than unencapsulated ones [46] and are less susceptible to phagocytosis [47]. However, the role of capsules in adherence is not clear. The present study showed no relationship between capsules and adherence since only two strains of the 16 examined showed microscopic evidence of capsule formation, and none had a macroscopic mucoid colonial morphology.

From the results of this study, we must conclude that while different *Staphylococcus aureus* strains show significant variation in their adherence to Hep-2 cells in vitro, there is no association between adherence and methicillin resistance, cell-wall bound protein A content, capsule formation or epidemic type. Specifically, we found no increase in adherence or protein A expression amongst the highly epidemic EMRSA strains EMRSA-15 and EMRSA-16.

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