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Discrimination between methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* using pyrolysis mass spectrometry and artificial neural networks

Royston Goodacre[a]*, Paul J. Rooney[b] and Douglas B. Kell[a]

[a]*Institute of Biological Sciences, University of Wales, Aberystwyth SY23 3DA, UK;* [b]*Bronglais General Hospital, Aberystwyth, SY23 1ER, UK*

Curie-point pyrolysis mass spectra were obtained from 15 methicillin-resistant and 22 methicillin-susceptible *Staphylococcus aureus* strains. Cluster analysis showed that the major source of variation between the pyrolysis mass spectra resulted from the phage group of the bacteria, not their resistance or susceptibility to methicillin. By contrast, artificial neural networks could be trained to recognize those aspects of the pyrolysis mass spectra that differentiated methicillin-resistant from methicillin-sensitive strains. The trained neural network could then use pyrolysis mass spectral data to assess whether an unknown strain was resistant to methicillin. These results give the first demonstration that the combination of pyrolysis mass spectrometry with neural networks can provide a very rapid and accurate antibiotic susceptibility testing technique.

Introduction

When a pathogen is isolated in a microbiology laboratory, the time taken for subsequent culture for identification and susceptibility testing may delay the administration of the most appropriate treatment. For routine clinical purposes, the ideal method for bacterial identification and antibiotic susceptibility testing would have minimum sample preparation, would analyse samples directly (i.e. would not require reagents), and would be rapid, automated, and relatively inexpensive.[1] Most of these requirements are met by spectroscopic solutions, the commonest such approach being pyrolysis mass spectrometry (PyMS). PyMS can identify an organism to species level in 2 min, but to date the conventional methods for analysing PyMS data have been insufficiently powerful to allow antibiotic susceptibility data to be extracted from the spectra.

PyMS first involves pyrolysis, the thermal degradation of complex material in an inert atmosphere or a vacuum. This causes molecules to cleave at their weakest points to produce smaller, volatile fragments called 'pyrolysate'. A mass spectrometer can then be used to separate the components of the pyrolysate on the basis of their mass-to-charge ratio (m/z) to produce a pyrolysis mass spectrum, which can then be used as a 'biochemical profile' or fingerprint of the complex material analysed. This method is sufficiently sensitive to detect changes at the level of the genome,[2] and within microbiology PyMS has largely been used as a taxonomic aid in the identification and discrimination of different micro-organisms.[3,4] It is now considered to be a valuable system for the rapid epidemiological typing of clinically significant pathogens.[5]

There has been a dramatic increase in the incidence of nosocomial infections caused by strains of *Staphylococcus aureus* that are resistant to multiple antibiotics, usually because of transfer (acquisition) of resistance genes.[6] Methicillin-resistant strains of *S. aureus* (MRSA) were first isolated in 1961 following the introduction of this β-lactam for the treatment of staphylococcal infections.[7] Their resistance results from the presence of a novel additional penicillin binding protein (PBP) PBP 2′.[8,9] PBP 2′ is encoded by the *mecA* gene, which is part of the chromosomal DNA *mec* sequence, a 30 to 40 kb piece of DNA the origin of which is unknown.[10]

The aim of this study was to use PyMS to examine a collection of 37 methicillin-susceptible (MSSA) and methicillin-resistant (MRSA) *S. aureus* strains. Cluster analysis and artificial neural networks were used to determine whether pyrolysis mass spectra could be used to discriminate these strains on the basis of their methicillin susceptibility.

*Corresponding author. Tel: +44-1970-621947; Fax: +44-1970-622354; E-mail: rrg@aber.ac.uk

Materials and methods

Organisms and cultivation

Twenty-two MSSA and 15 MRSA were used in this study. These cultures were chosen to represent a diverse range of strains. Moreover, they possessed a wide range of resistance to other antibiotics (data not shown). The National Collection of Type Cultures set of 22 propagating strains for phage typing were used as examples of MSSA.[11] Three recent clinical isolates of MRSA (H34, E9/95/94, and E18/103/94) were supplied by Bronglais General Hospital along with a national standard MRSA (NCTC 10042), whilst the remainder were supplied by Dr Judith Richardson (Laboratory of Hospital Infection, Central Public Health Laboratory, London, UK). Details of the strain designations, phage group, and resistance or susceptibility to methicillin are given in the Table.

Strains were cultured on Mueller–Hinton agar (Oxoid-Unipath Ltd, Basingstoke, UK) plus 2% NaCl, which favours the expression of PBP 2′,[8,9] and incubated aerobically for 16 h. The bacteria were carefully removed from the agar surface with a plastic loop and suspended in physiological saline (0.9% NaCl) to approximately 20 mg/mL. The samples were then ready for analysis by PyMS.

Pyrolysis mass spectrometry (PyMS)

Five microlitres of the above samples was evenly applied to iron–nickel foils to give a thin uniform surface coating. Prior to pyrolysis the samples were oven dried at 50°C for 30 min. Each sample was analysed in triplicate. The pyrolysis mass spectrometer used was a Horizon Instruments PYMS-200X (Horizon Instruments Ltd, Ghyll Industrial Estate, Heathfield, East Sussex, UK).[12,13] The sample tube carrying the foil was heated, prior to pyrolysis, at 100°C for 5 s. Curie-point pyrolysis was at 530°C for 3 s, with a temperature rise time of 0.5 s. The data from PyMS were collected over the *m*/*z* range 51 to 200 and may be displayed as quantitative pyrolysis mass spectra (e.g. as in Figure 1). The abscissa represents the *m*/*z* ratio whilst the ordinate contains information on the ion count for any particular *m*/*z* value between 51 and 200. Data were normalized as a percentage of total ion count to remove the most direct influence of sample size *per se*.

Multivariate cluster analysis

Canonical variates analysis (CVA) (also referred to as 'discriminant function analysis' (DFA)) is a multivariate statistical technique that separates objects (samples) into groups or classes by minimizing the within-group variance and maximizing the between-group variance.[14,15]

Before CVA was employed, principal components analysis (PCA) was used to reduce the dimensionality of the data and only those principal components (PCs) whose eigenvalues accounted for more than 0.1% of the total variance were used. When the first few PCs represent a large proportion of the total variance, it is likely that further axes generated will result from random noise in the data; these PCs can be ignored without reducing the amount of useful information representing the data, since each PC is now independent of (uncorrelated with) any other PC.[15,16,17] CVA then separated the objects (samples) into groups on the basis of the retained PCs and the *a priori* knowledge of the appropriate number of groupings;[14,18,19] the *a priori* groups here are the known triplicate pyrolysis mass spectra and so do not bias the analysis in any way. The objective of CVA is to maximize the ratio of the between-group to within-group variance; therefore a plot of the first two canonical variates (CVs) displays the best two-dimensional representation of the *group* separation. To effect CVA the normalized data were processed with the GENSTAT package[20] run under Microsoft DOS 6.22 on an IBM-compatible PC.

Next, a percentage similarity matrix was constructed by transforming the Mahalanobis distance[15] between *a priori* groups in canonical variates analysis with the Gower similarity coefficient S_G.[21] Finally, hierarchical cluster analysis was employed to produce a dendrogram, using average linkage clustering.[22]

Creation of training and test data sets for artificial neural network analyses

It is well known that if the number of weights in a neural network is significantly higher than the number of exemplars in the training set then overfitting can more easily occur.[23,24] Therefore, to obey the parsimony principle, as described by Seasholtz & Kowalski,[23] the next stage was to reduce the number of inputs to the ANNs. PCA is an excellent dimensionality reduction technique,[17] and the first five PC scores from the averaged normalized triplicate pyrolysis mass spectra were used as the input data (these accounted for 90.4% of the total variance). The first five PCs were used because when too few PCs are used, not enough information is present, and when more PCs are employed, the later PCs are likely to contribute only noise to the model, thus increasing the probability of chance correlations between input and output data.

In addition, it is important that the training data encompass the full range under study[24,25,26] since, although supervised methods are excellent at being able to interpolate, they are likely to give poor estimates outside their 'realm of knowledge', i.e. they cannot extrapolate sufficiently well. Since the 37 strains of *S. aureus* encompassed a diverse range of epidemiologically distinct strains it was imperative that the training set of MSSA and MRSA represented multidimensional space sufficiently well to allow interpolation.

'Duplex' is a method for choosing an optimal split between training and test data sets;[27] an extension to this methodology called 'Multiplex' has been developed in-

Table. Identity of the *S. aureus* used in the test set and training set as judged by artificial neural networks

Set	*S. aureus* strain	Phage group of strain[a]	Type[b]	MLPs estimate[c]
Training	PS 47	I + III	S	0.00
	PS 54	III	S	0.04
	PS 84	III	S	0.01
	PS 71	II	S	0.00
	PS 55	II	S	0.00
	PS 95	95	S	0.00
	PS 3C	II	S	0.00
	PS 52	I	S	0.00
	PS 29	I	S	0.00
	PS 80	I	S	0.00
	PS 83A	III	S	0.00
	PS 85	III	S	0.00
	PS 77	III	S	0.04
	PS 75	III	S	0.03
	PS 53	III	S	0.01
	PS 42E	III	S	0.06
Test	PS 81	I	S	0.00
	PS 6	III	S	0.00
	PS 52A/79	I	S	0.00
	PS 3A	II	S	0.00
	PS 96	V	S	0.00
	PS 94	V	S	0.00
Training	CRF 631 PS	I	R	1.00
	CRF 634 PS	III	R	0.98
	ST 84 6255	III	R	0.95
	ST 85 1774	NT	R	0.97
	CRF 627 PS	III	R	1.00
	CRF 621 PS	NT	R	0.97
	ST 84 6144	NT	R	1.00
	ST 84 6983	–	R	1.00
	CRF 619 PS	III	R	1.00
	H34	NT	R	0.97
	E18/103/94	III	R	0.95
Test	CRF 633 PS	III	R	0.99
	ST 85 3566	NT	R	1.00
	E9/95/94	NT	R	0.94
	NCTC 10442	III	R	0.99

[a] NT, not typeable; – , no data available.
[b] Methicillin susceptibility.
[c] The MSSA strains were coded as 0 and MRSA as 1 at the output node. The values shown are the average of ten neural network runs; the standard deviations for these values were all ≤ 0.02. The MLPs typically took 1.3×10^3 ($\pm 4 \times 10^1$) epochs to train.

house (Jones, A. *et al.*, personal communication). Briefly, this method starts by placing the two most separated samples into the training set. It then places the next two most separated remaining samples into the test set. This is performed iteratively until all samples have been split. This ensures that the training set range covers the test set range, and that both sets are representative. The first five PC scores from the pyrolysis mass spectra were sorted using Multiplex so that the training and test data were split in the ratio 3:1. Data may be split on both the X matrix (PC scores) and the Y matrix (bacterial type); so as not to bias the partitioning process, data were split on the X matrix only.

Artificial neural networks (ANNs)

All ANN analyses were carried out with a user-friendly, neural network simulation program, NeuFrame version 1,1,0,0 (Neural Computer Sciences, Lulworth Business Centre, Nutwood Way, Totton, Southampton, UK), which runs under Microsoft Windows NT on an IBM-compatible PC. In-depth descriptions of the *modus operandi* of this type of multilayer perception (MLP) analysis are given elsewhere.[12,28,29,30]

For training the ANNs, each of the inputs consisted of the first five PC scores from the averaged normalized triplicate pyrolysis mass spectra. These were derived from the data as split above using the multiplex program (details are given in the Table) and each was paired with one of the desired outputs. These were binary encoded such that the MSSA strains were coded as 0 and MRSA coded as 1 at the output node. These training pairs collectively made up the training set. The structure of the ANN used in this study was fully connected and consisted of three layers: five input nodes, one output node, and one 'hidden' layer containing three nodes (i.e. a 5–3–1 architecture). Before training commenced, the values applied to the output nodes were normalized between 0 and 1. The scaling regime used for the input layer was to scale each node such that the lowest PC was set to 0 and the highest to 1. For the present purposes these ANNs were trained to a root mean squared (RMS) error of 0.025, which typically took 1.3×10^3 epochs to train ($\pm 4 \times 10^1$ epochs). An 'epoch' is a complete calculation in the network, when all of the training data have been presented to the ANN once. This process was conducted ten times to observe whether training was reproducible and also to use the 'committee' approach for prediction,[24] averaging the outputs from the ten 5–3–1 ANNs.

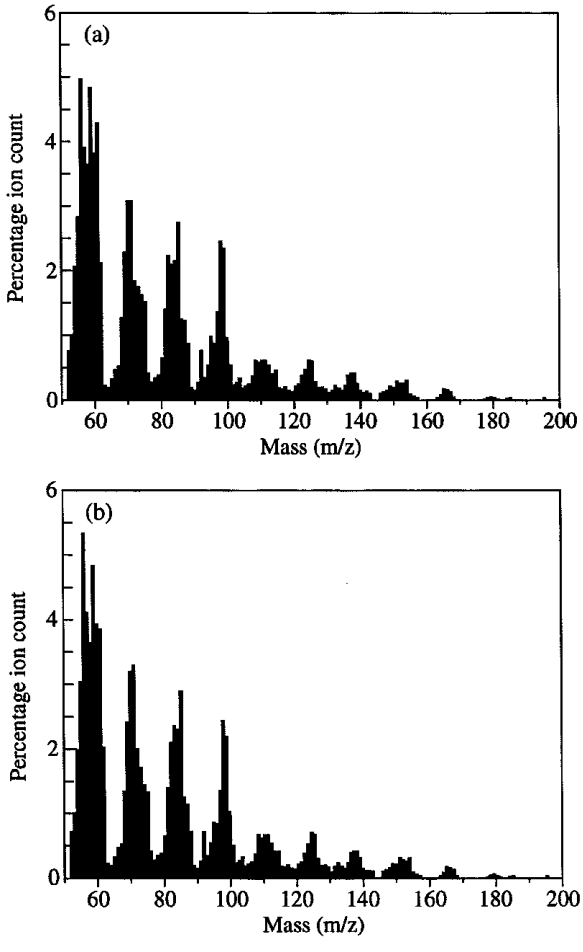

Figure 1. Normalized pyrolysis mass spectra of (a) methicillin-susceptible *S. aureus* strain PS 54 and (b) methicillin-resistant *S. aureus* strain E9/95/94.

Results and discussion

Typical normalized PyMS spectra for methicillin-susceptible *S. aureus* strain PS 54 and methicillin-resistant *S. aureus* strain E9/95/94 are shown in Figure 1. Although there was very little qualitative difference between these spectra, small complex quantitative differences between the spectra were observed. Such spectra cannot be interpreted by the naked eye and illustrate the need to employ multivariate statistical techniques for the analysis of PyMS data.

After collection of the pyrolysis mass spectra, each of the 37 strains, each represented by three replicate spectra, was coded to give 37 individual groups (see Table) and analysed by CVA. The resulting ordination plot is shown in Figure 2, where the first three CVs are shown, which account for respectively 48.2, 21.5, and 11.8% (80.5% total) of the total variance. The coding in this plot is simply for whether the strain is MSSA (indicated by a 'S') or MRSA ('R'), and shows that CVA cannot be used to cluster these bacteria according to whether they are resistant or susceptible to methicillin—because two distinct groups were not formed.

An alternative way of viewing the relationship between these 37 *S. aureus* strains is to perform hierarchical cluster

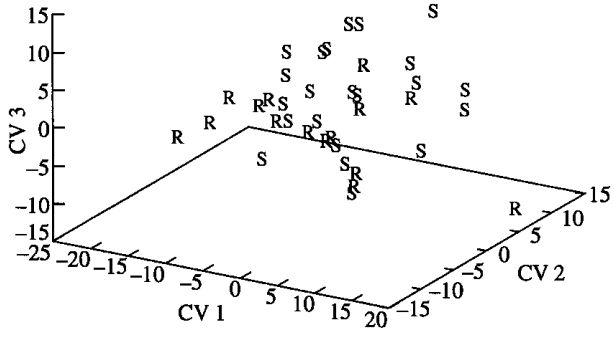

Figure 2. Pseudo-3D canonical variates analysis plot based on PyMS data analysed by GENSTAT, showing the relationship between the 37 *S. aureus* strains. The first 17 PCs were used as the inputs for the CVA algorithm and accounted for 99.4% of the variance. 'S' refers to MSSA and 'R' to MRSA.

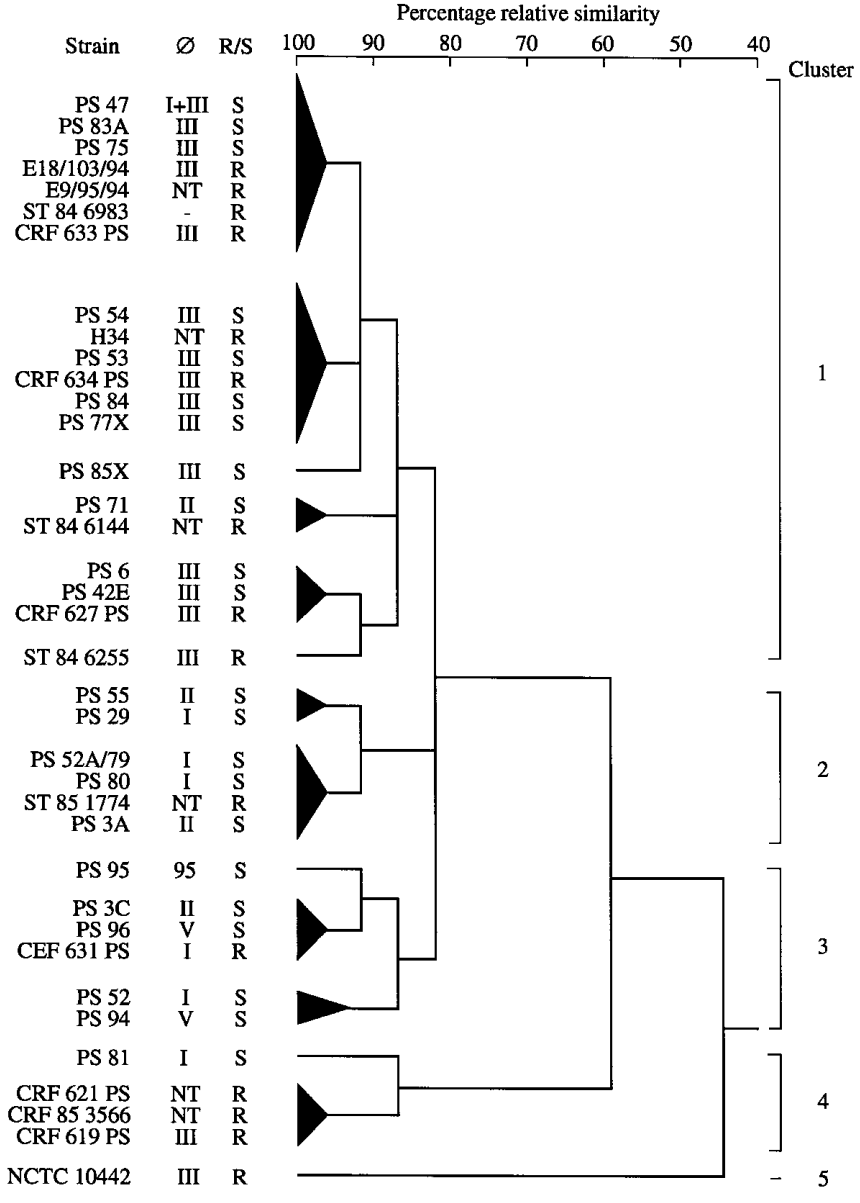

Figure 3. Dendrogram representing the relationships between the 37 *S. aureus* strains based on PyMS data analysed by GENSTAT. The first 17 PCs (accounting for 99.4% of the total variance) were used for CVA before construction of the similarity matrix and dendrogram. 'Ø' refers to the phage group of the strain. 'S' refers to MSSA and 'R' to MRSA.

analysis (HCA). The dendrogram produced from HCA is shown in Figure 3, where it can be seen that at 85% relative similarity the bacteria were grouped into five clusters. Cluster 1 is a heterogeneous group of 11 MSSA and nine MRSA; 15 of 20 strains were group III. Cluster 2 consisted of five MSSA, three of phage group I and two of group II, along with a single untypeable MRSA. Cluster 3 contained six strains, five MSSA and one MRSA of phage group I; both propagating strains of phage group V were in this cluster. Cluster 4 contained three strains of MRSA, one of group III and two that were untypeable, and PS 81. Cluster 5 contained only MRSA NCTC 10442. These results indicate that the major difference between the PyMS spectra of the 37 strains was due to their phage type, and that they did not cluster according to their methicillin susceptibility.

In other experiments, CVA was used to analyse all 37 strains, where all the MSSA were coded as a single group and all the MRSA as another group. The first canonical variate displayed 100% of the total variation, because two groups can be separated in one dimension. It is possible to depict this as a histogram in which the abscissa represents the canonical variate distance and the ordinate contains information on the number of samples that appear in that area (Figure 4). It is obvious from this histogram that MRSA and MSSA were not separated and there was a great deal of group overlap; moreover, for two groups of

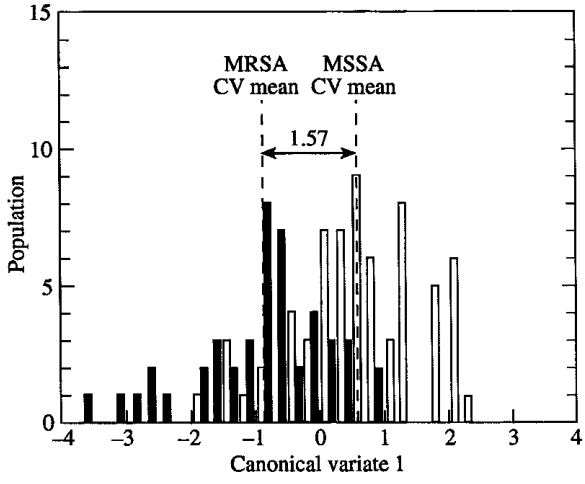

Figure 4. Histogram of canonical variate 1 based on PyMS data analysed by GENSTAT, coded so that all the MSSA strains (□) were coded as a single group and all the MRSA (■) as another group. In this histogram the abscissa represents the canonical variate distance for CV1 and the ordinate contains information on the number of samples that appear in that area.

pyrolysis mass spectra to be significantly discrete, the CVA group means must be separated by more than 3.84 canonical variate units, which represents the 95% confidence limit constructed round each mean by the χ^2 distribution on one degree of freedom.[15] For the separation of MRSA from MSSA this distance was only 1.57 units. Using this linear method of analysis it was therefore impossible to separate these two classes on the basis of their pyrolysis mass spectra.

The above methods of multivariate analysis, which separate spectra by linear transformation of the data, could not yield any reliable information on the resistance or sensitivity of *S. aureus* to methicillin. The next stage was therefore to examine the ability of artificial neural networks (ANNs), a supervised method that can uncover non-linear relationships between the two classes of bacteria and which is greatly superior to clustering techniques in classification problems of this type.[31,32]

As detailed above, the 37 strains encompassed a diverse range of epidemiologically distinct strains and it was thus imperative that the training set for the ANNs should represent the PyMS multidimensional data space sufficiently well to allow interpolation, and that the range of the test set was enclosed by the training set. Therefore, the program Multiplex was used to split the data equally into training and test sets.

ANNs were trained with the first five PC scores from the averaged normalized triplicate pyrolysis mass spectrum from the training set; the 16 MSSA were coded 0 at the output node and the 11 MRSA were coded 1. The 5–3–1 ANNs were trained using the standard back-propagation algorithm, and the effectiveness of training was expressed in terms of the RMS error between the actual and the desired outputs; training was stopped after the RMS error had reached 0.025. Training was effected ten times, using randomized, small initial values for the starting weights; the ten learning curves were seen to superimpose (data not shown) and it was clear that, despite the randomized starting connection weights, training was executed (i.e. the error surface in weight space was negotiated) in a reproducible manner. Moreover, these ANNs typically took 1.3×10^3 epochs to train to an RMS error of 0.025 within a spread of only $\pm 4 \times 10^1$ epochs.

When training had ceased (as determined by the attainment of an RMS error of 0.025 averaged over the training set), the ten neural networks were interrogated (challenged) with the normalized ion intensities of the pyrolysis mass spectra from both data sets. Not surprisingly, the network's estimate of the resistance or susceptibility to methicillin of the training set was the same as those known in all ten trainings (see Table). The results of the ANN's final analyses of the unknown test set is also shown in the Table. This table is the average of the ANN's predictions for each of the replicates of the 37 strains; small standard deviations were calculated (the largest was only 0.017), indicating that training was indeed reproducible. Rather than using a simple crisp identification criterion (if the output is > 0.5 then the strain is a MRSA; if the output is < 0.5 then the strain is a MSSA), a correct identification was made as follows: for MRSA the output must be $\geqslant 0.8$ and for MSSA it must be $\leqslant 0.2$. This procedure allows a more rigid classification to be used, since if any output is close to 0.5 ($\pm$ 0.3) the ANN would be 'undecided' about the identification, and hence unable to discriminate that bacterial sample sufficiently well.

It is evident from the Table that the ANN had assessed correctly each *S. aureus* strain from the unseen test set, as MRSA or MSSA. All four MRSA strains scored > 0.93, and all six MSSA scored < 0.01. These results show that there were neither false negatives nor false positives.

It is likely that these ANNs were able to generalize well (i.e. to assess correctly the methicillin susceptibility of these staphylococci) because the training set contained a wide assortment of *S. aureus* that were representative of the problem domain. Indeed, in other runs (data not shown) the training and test sets were chosen randomly, and typically three out of ten strains in the test sets were classed incorrectly. It was likely that in these instances the training sets contained blind spots and there were gaps in the problem domain which led to incorrect identifications. Since a wide assortment of genotypically, and hence phenotypically, diverse staphylococci were studied, the ANNs had learnt to extract the (bio)chemical information buried in the mass spectra which conveyed whether the *S. aureus* strains were susceptible or resistant to methicillin, irrespective of the epidemiology of the strains under study. It is likely that this phenetic information is from the PBP 2′ protein encoded by the *mecA* gene; however, currently it is impossible to extract this knowledge from ANNs.

In addition, ANNs are a well known means of uncovering complex, non-linear relationships in multivariate data, whilst still being able to map the linearities.[24,30] It is likely that part of the reason why CVA and HCA failed to discriminate the MRSA from the MSSA is because they rely on linear (orthogonal) transformations of the raw multivariate data; they cannot provide the truly best analytical discriminations, and thus achieved only limited success. In order to investigate this point further, another linear multivariate statistical model, partial least squares (PLS), was implemented as outlined by Martens & Næs,[33] with the same training and test sets as used above. However, PLS could identify only one of the four MRSA strains and five of the six MSSA in the same test set when using PCs as inputs, and the identifications were difficult to interpret since they were less quantized (i.e. very few of the predictions were very near 0 or 1). This would suggest that the non-linearity of the system is important, as is the use of a supervised method *per se*, in determining the ability to discriminate MRSA from MSSA from their pyrolysis mass spectra. However, while it is well known that ANNs are an excellent means of classifying groups of objects, as we have demonstrated here, it is also well known that it is very difficult to assess the information used by an ANN in producing its internal model.

In conclusion, PyMS with cluster analysis showed that the major difference between the 37 *S. aureus* strains studied resulted from the phage group and not from their resistance or susceptibility to methicillin. However, ANNs could be trained to assess whether an unknown strain were resistant to methicillin, and there were neither false negatives nor false positives. ANNs have proved advantageous in the analysis of PyMS data, and these mathematical techniques based on artificial intelligence have now been adopted by an increasing number of workers for the identification of bacteria from their PyMS spectra.[1] PyMS is a physico-chemical method that measures the bond strengths of molecules and gives quantitative information about the total biochemical composition of a sample. Therefore PyMS signatures may change depending on which media the bacteria are cultivated on and, to remove any effects of variable phenotype, all strains under study are usually incubated under identical conditions. However, we have shown that the PyMS spectra of *Carnobacterium* spp. were unaffected by widely different culture ages grown at 30°C for 24, 48 and 72 h,[34] which implies that changes in phenotype may not always be as significant as was thought previously. In addition, although PyMS, like other analytical tools, suffers from long-term reproducibility problems which limit its use to the typing of short-term outbreaks where all micro-organisms are analysed in a single batch,[3] other associative ANNs can be used as signal-processing elements to effect the transformation of data acquired on one day to those acquired on a different date.[35,36] As the problems of long-term reproducibility are overcome, PyMS may move closer to application in clinical laboratories for rapid identification and antibiotic susceptibility testing of bacteria and fungi. We conclude that the application of artificial neural network methods can be used to extend the role of PyMS analyses to more subtle physiological differences between strains of the same species of bacteria and, in this case, for rapid and accurate methicillin susceptibility testing of *S. aureus*.

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