The use of Raman spectroscopy in the epidemiology of methicillin-resistant *Staphylococcus aureus* of human- and animal-related clonal lineages

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Abstract

In order to perform a cost-effective search and destroy policy for methicillin-resistant *Staphylococcus aureus* (MRSA), a quick and reliable typing method is essential. In an area with a high level of animal-related MRSA ST398, pulsed field gel electrophoresis (PFGE) typing and *spa*-typing are not sufficient to discriminate between co-incidental findings and true transmission of MRSA. This study is the first to retrospectively show the performance of Raman spectroscopy in 16 well-documented outbreaks. We analysed 525 isolates, 286 MRSA ST398 and 239 from other PFGE clusters with Raman spectroscopy. When epidemiologically linked isolates from the outbreaks were analysed with PFGE as the reference standard, Raman spectroscopy correctly identified 97% of cases that were indistinguishable from the index case. With Raman cluster analysis, the most dominant distinction was between MRSA ST398 and other MRSA of human clonal lineages. Within MRSA ST398, 22 different Raman clusters were identified. Raman typing correctly identified an ST398 (*spa* type t567) outbreak in a hospital setting. No direct correlation was observed between Raman clusters and *spa* types. We conclude that Raman spectroscopy is a quick and reliable method of MRSA typing, which can be used in outbreak settings and it is comparable to PFGE, with the added advantage that PFGE non-typeable isolates can also be readily typed using the same sample preparation protocol.

Keywords: Methicillin-resistant *Staphylococcus aureus*, Raman spectroscopy, ST398, *Staphylococcus aureus*, spa typing
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Introduction

In the Netherlands the prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) is low. In blood cultures positive for *S. aureus*, only 1% is methicillin resistant and carriage rates in the general population are between 0.08 and 0.03% [1,2]. This is thought to be due to both a restrictive use of antibiotics and an active MRSA search and destroy policy. This policy involves screening of all patients transferred from foreign hospitals, screening of all contacts of MRSA-positive patients, including healthcare workers and fellow patients, and, since July 2006, screening of people in contact with livestock (pigs and veal calves) (http://www.wip.nl).

In the south-east of the Netherlands (the catchment area of the PAMM laboratory), the number of newly identified MRSA-positive individuals increased from 16 per year between July 2002 and July 2006 to 148 per year between July 2006 and December 2008. Eight-one per cent of this increase is due to MRSA of multilocus sequence type (MLST) ST398 (Methicillin-resistant Staphylococci in Animals: Veterinary and Public Health Implications, London, 2009, S3:4), a type of MRSA associated with contact with livestock. Of this 81%, the majority came from targeted screening (98 per year, 74%) but 7% was due to unexpected cases.

An unexpected MRSA case in a hospital or nursing home, will lead to a screening of close contacts of the index patient. If more MRSA-positive individuals are found, an

outbreak investigation will be started but the manner and magnitude of the investigation will depend on whether or not there was transmission. Consequently, the need is felt for a reliable and preferably fast typing method for all MRSA, including MLST ST398, in order to make timely decisions on the outbreak investigation regime. The difficulty with MRSA of this sequence type, is that it is non-typeable with standard pulsed field gel electrophoresis (PFGE) using *Sma* I restriction [3], which was the standard method in the national reference laboratory in the Netherlands (RIVM) until 2007. As of January 2008, *spa*-typing is used but because most animal-related isolates belong to a small number of closely related *spa*-types, this method does not have the discriminatory power required [4]. Raman spectroscopy is an optical method that relies on spectroscopic fingerprints that represent the complete molecular composition of a microorganism. The method was recently shown to be effective for strain typing of staphylococci [5]. In this study we describe the use of Raman spectroscopy on isolates from screening cultures and clinical isolates of both PFGE typeable and non-typeable strains.

Materials and Methods

Isolate collection

The PAMM Laboratory for Medical Microbiology has an adherence area of 800 000 people in the south-east of the Netherlands and provides services to four hospitals and the general practitioners and nursing homes in the area. This area also has the highest density of pig farms in the Netherlands [6]. A total of 525 MRSA-positive individuals, whose isolates were stored and PFGE typing data known, were identified in the laboratory database between July 2002 and December 2008. All these isolates were confirmed as MRSA by *femA*/*mecA* PCR. PFGE typing results and *spa* types were obtained from the national reference laboratory (RIVM, Bilthoven). Of these 525 isolates, 286 were non-typeable by PFGE and/or belonged to *spa*-types corresponding with MRSA ST398.

Clinical data

Clinical data for the isolates from this study were collected from the MRSA surveillance records from the infection control departments of the hospitals, the clinical consultation form in the laboratory information system and from patient charts. For each isolate the origin was noted: clinical (unexpected MRSA) or obtained during targeted screening. For all targeted screening isolates the reason for screening was included, as well as known contacts between positive patients and/or healthcare workers.

Raman spectroscopy

Raman measurements and analyses were performed in blinded fashion; for example, isolates were numbered and PFGE and *spa* typing results and clinical data were supplied only after measurements and cluster analysis were carried out.

For Raman measurements, all isolates were grown overnight on Trypticase Soy agar (TSA; Becton Dickinson, Franklin Lakes, NJ, USA). Samples were prepared as described previously [5]. Briefly, after a 20-h culture, biomass was suspended in water, transferred to a sample carrier and allowed to dry. Samples were measured on the advanced prototype of the SpectraCell*RA*® bacterial strain analyzer from River Diagnostics BV (Rotterdam, the Netherlands). For 24 samples, the system throughput time was 2 h and this involved about 30 min of hands-on time.

Samples were measured over a period of 2 months. After 1 year, 45 samples were repeated together with a new batch of samples to determine the reproducibility over a longer period of time.

Data analysis

Raman types were determined using Wards cluster algorithm with a fixed cut-off established at 99.95% similarity. This cut-off was based on the lowest similarity observed between three full biological replicates (independent repeat Raman measurements of freezer stock) of 116 isolates. Isolates grouped in a cluster were assigned a unique Raman type. Raman types were compared with PFGE data and a retrospective analysis was performed to see whether Raman typing data would have provided sufficient data to make adequate decisions in the context of an outbreak investigation.

Reproducibility over time was calculated as the percentage of isolates for which the replicate measurements are combined in the same cluster in the dendrogram.

Results

From a total of 525 isolates, 286 were non-typeable by PFGE (NT-MRSA). The remaining 239 isolates belonged to 52 different PFGE clusters and 40 Raman types.

Epidemiological data identified 16 potential outbreaks and/or cases of suspected transmission of typeable MRSA, with a total of 142 isolates. The outbreaks occurred in nursing homes, hospitals and families. Table 1 shows the main characteristics of the outbreaks with regard to the number of isolates and correspondence between PFGE clusters and Raman types.

Of the 142 isolates, 127 (89%) cases were identified where the PFGE cluster was identical to that of the index case. In 123 (87%) cases the same applied for the Raman type. When PFGE is regarded as the reference standard, Raman spectroscopy would come to identical conclusions in 123 out of 127 cases (97%). Furthermore, in 18 cases in which the PFGE type was different from the index, the same applied with regard to Raman type (Table 1).

In retrospect, in the six outbreaks where PFGE showed identical types to the index case but Raman clusters were different, this would not have led to a termination of the outbreak investigation because in the same round of screening identical isolates were found as well.

Raman and NT MRSA

It was very interesting to see that the most dominant distinction between the isolates was that between PFGE typeable and NT isolates (Fig. 1). This finding confirms the genetic evidence that the NT isolates are a subpopulation within the *S. aureus* species, using phenotypic data generated by Raman typing. At the first branch in the dendrogram, 512 of 525 (98%) isolates were correctly grouped in the typeable or NT cluster. Eight PFGE non-typeable isolates were designated a Raman type associated mainly with PFGE-typeable isolates. The reason could be a technical failure to produce restriction fragments. In contrast, five isolates were typeable by PFGE but were designated a Raman type associated with non-typeable isolates. This resulted in a specificity 98% and sensitivity of 97% for distinguishing PFGE NT and ST398 isolates.

Two hundred and eighty-six PFGE non-typeable isolates were divided into 22 Raman types. The three most predominant types are 30 (n = 87 isolates), 26 (n = 43) and 28 (n = 43). Seven isolates had a unique Raman type. In order to asses the clinical relevance, data were compared with known epidemiological relationships between patients

TABLE 1. Description of outbreak involving PFGE typeable isolates

Outbreak number	Number of isolates	PFGE types (number of isolates)	Raman types (number of isolates)	Outbreak description with comments on Raman clusters
2002-1	18	158[a] (n = 18)	9[a] (n = 18)	6 patients and 12 HCWs, no discrepancies between PFGE and Raman typing
2002-2	36	158[a] (n = 34) 113 (n = 2)	9[a] (n = 32) 26 (n = 1) 31 (n = 1) 6 (n = 1) 22 (n = 1)	26 patients and 10 HCWs. Two patients with both a different PFGE and Raman from the index case. Two more discrepant Raman clusters, 26 and 31, were found in the long-term follow-up of the outbreak (3 months and 9 months after initial screening started, respectively)
2003-1	11	71[a] (n = 6) 271 (n = 1) 115a (n = 2) 16d (n = 1) 209a (n = 1)	14[a] (n = 7) 13 (n = 2) 31 (n = 1) 23 (n = 1)	7 patients with identical Raman (14) and 6 with identical PFGE (71). Other discrepant PFGE types also had different Raman types.
2003-2	6	55[a] (n = 4) 248 (n = 2)	23[a] (n = 4) 29 (n = 2)	3 patients and one HCW positive. Family members of the HCW positive with both different PFGE 248 and Raman 29
2004-1	5	55[a] (n = 2), 305 (n = 1) NT (n = 2)	23[a] (n = 3) 30 (n = 2)	3 family members identical Raman types but two PFGE types (55, 305). Screening of contact patients showed different Raman and PFGE types
2005-1	2	137B[a] (n = 2)	24[a] (n = 2)	Transmission between patient and HCW
2006-1	12	15[a] (n = 12)	31[a] (n = 11) 33 (n = 1)	2 patients and 10 HCWs. Discrepant Raman found in only patient (beside index case) in outbreak
2006-2	4	113[a] (n = 4)	11[a] (n = 3) 10 (n = 1)	Index patient and family member and HCW with identical isolates. One other HCW with a PFGE identical but Raman discrepant iso late. This isolate had a different resistance pattern: ciprofloxacin, clindamycin S, fusidic acid R, while the other 3 isolates had identi cal resistance patterns
2007-2	4	113[a] (n = 4)	10[a] (n = 3) 28 (n = 1)	One patient with a discrepant Raman cluster. Identical resistance patterns
2007-3	3	218a[a] (n = 2) 71a (n = 1)	25[a] (n = 2) 5 (n = 1)	Family members with MRSA. One member after 1 month both dif ferent PFGE and Raman cluster
2007-5	3	71a 65[a] (n = 3)	5 17[a] (n = 3)	Index positive (PFGE 71a, Raman 5), screening of contacts, 1 HCW positive with different PFGE/Raman, 2 contacts of this HCW with PFGE 65, Raman 17
2007-6	2	113[a] (n = 1) NT (n = 1)	10 (n = 1) 4 (n = 1)	Index patient with an NT, Raman 4 isolate, HCW with PFGE 113/ Raman 10. Interpreted as coincidental finding, screening stopped after one ring
2007-7	4	23[a] (n = 3) NT (n = 1)	27[a] (n = 3) 30 (n = 1)	Two patients and one HCW with same strain, one HCW with an NT isolate that also has a different Raman type (30)
2007-8	23	65[a] (n = 23)	17[a] (n = 23)	11 patients and 12 HCWs positive with identical isolates
2007-9	6	113[a] (n = 6)	11[a] (n = 6)	6 patients in nursing home
2007-10	3	28[a] (n = 3)	25[a] (n = 3)	3 MRSA infections in one family
Total	142	127[b]	123[b]	

[a]Indicates corresponding PFGE and Raman type to the index case.
[b]Total number of isolates recognized as being identical to index case.
HCW, healthcare worker; PGFR, pulsed field gel electrophoresis.

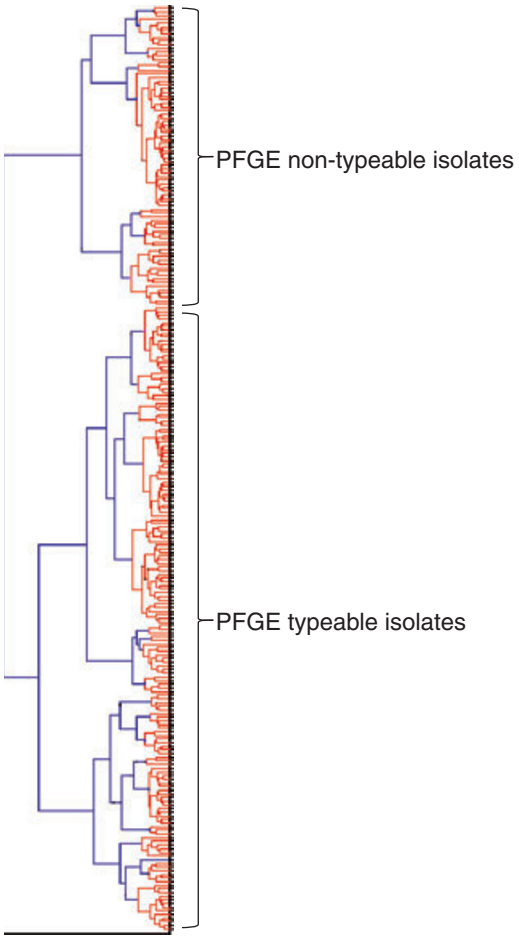

FIG. 1 Dendrogram obtained from a cluster analysis on isolates in this study. The figure shows that the most dominant distinction is that between the pulsed field gel electrophoresis typeable and non-typeable isolates.

(Table 2). In the included hospital outbreak [7] all nine isolates belonged to one Raman type (number 4).

Within families exposed to livestock, different Raman types were found. This also occurred in subsequent cultures of individual patients. Recently we have shown that Raman typing was able to reliably discriminate between multiple strains colonizing a single patient [8].

It is difficult to attach significance to this finding here, because it is uncertain whether people in contact with livestock become carriers because of a one-time acquisition of the MRSA strain or because they have a continuous exposure to the source. There is some evidence for the latter because carriage of MRSA ST398 is short lived in people with a one-time exposure [9]. In addition, different strains can be found if livestock is bought from different sources.

Spa typing and Raman types

For 255 of the NT isolates, *spa*-typing was available. There was no direct correlation between the two most common *spa* types, t108 and t011, and any of the Raman clusters. However, 21 of 27 isolates (77%) with *spa* type t567 belonged to Raman type 4 and no other *spa* types were associated with this Raman type.

Reproducibility

To asses reproducibility over time and after freezing and thawing, 20 non-typeable and 26 PFGE-typed isolates were tested at two different points in time. Reproducibility was 95% (45/46 isolates having replicates in the same cluster).

TABLE 2. Description of outbreak involving PFGE non-typeable isolates (MSRSA ST398)

Outbreak number	Number of Isolates (*n*)	*spa* types (number of isolates[b])	Raman types (number of isolates)	Outbreak description with comments on Raman clusters
2006-3	2	PFGE 55 (*n* = 1) t567 (*n* = 1)	29[a] (*n* = 2)	Transmission from mother to child. Isolates with an identical resistance pattern
2007-1	2	t011[a] (*n* = 1) t108 (*n* = 1)	28 (*n* = 1) 18 (*n* = 1)	Contact screening around patient, one HCW positive, different type of MRSA
2007-4	9	t567[a](*n* = 9)	4[a] (*n* = 9)	Outbreak in hospital [7]
2007-11	5	t034[a] (*n* = 4) t011 (*n* = 1)	30[a] (*n* = 2), 29 (*n* = 1), 15 (*n* = 1) 19 (*n* = 1)	Family of five people living on calf farm
2007-12	2	t108[a] (*n* = 2)	30[a] (*n* = 2)	Family members, pig farm
2008-1	3	t011[a] (*n* = 1) t567(*n* = 2)	12 (*n* = 1) 4 (*n* = 1), 20 (*n* = 1)	Possible transmission in nursing home. Index patient with t011, 2 HCWs with t567, one had contact with horses but not calves and/or pigs
2008-2	2	t011[a] (*n* = 2)	28[a] (*n* = 2)	2 isolates from one patient with different resistance patterns
2008-3	2	t108[a] (*n* = 1) t011 (*n* = 1)	30 (*n* = 1) 12 (*n* = 1)	2 isolates from one patient with different resistance patterns
2008-4	2	t011[a] (*n* = 2)	18 (*n* = 1) 26 (*n* = 1)	2 isolates from one patient with different resistance patterns
Total	28	20	17	

[a]Indicates *spa*- and Raman type corresponding to the index case.
[b]Total number of isolates recognized as being identical to index case.
HCW, healthcare worker.

Discussion

In the context of an active search and destroy policy, rapid MRSA typing methods are important. When an MRSA is found in a patient, a first ring of contacts including patients and healthcare workers is screened (direct contacts of the index case). If other individuals are found carrying the same strain, the net is thrown wider and a second or even a third ring is screened (indirect contact with index). If the other MRSA isolates belong to a different strain (e.g. have a different PFGE or Raman type), a limited circle of people will be screened around this new index. Therefore a rapid and reliable typing method is essential to distinguish between transmission and a coincidental finding and to limit the screening efforts and associated costs. In this study Raman typing had a 97% similarity compared with typing by PFGE. The advantage of Raman spectroscopy is that it is a fast method and less labour intensive than PFGE, a significant advantage in outbreak settings. Reproducibility of Raman results over a period of 1 year was good: 95% (45/46 isolates showing replicate samples in the same Raman cluster).

Furthermore, Raman typing was able to divide so-called non-typeable MRSA into 22 distinct types. Raman typing correctly identified an ST398 (*spa* type t567) outbreak in a hospital setting. The different types seen in families could be due to the high discriminatory power of the Raman method or might mirror the fact that families harbour different types of MRSA due to the exposure to a common source (livestock) that might harbour different types of MRSA. The fact that there is a lack of clear correlation between Raman and *spa*-typing can be explained by the fact that a number of the closely related *spa*-types are found within the NT MRSA and *spa*-typing appears not to have a high enough discriminatory value for epidemiology within ST398, which is not unexpected because *spa*-typing involves repeats in one gene.

In order to type ST398 strains, modified PFGE procedures have been documented as well as typing by multiple Loci VNTR (variable number of tandem repeats) Analysis (MLVA) [4,10]. It was shown that different *spa*-types can occur within the same MLVA cluster and *vice versa* [4]. It would be interesting to compare these methods with Raman typing.

Conclusions

Raman spectroscopy is a quick and reliable method of MRSA typing that can be used in outbreak settings and is comparable to PFGE, with the added advantage that PFGE non-typeable isolates can also be readily typed using the same sample preparation protocol. Isolates with a correlation coefficient of 99.95% or higher should be considered identical, but more research is needed to establish which isolates should be considered closely related.

Transparency Declaration

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