Research articles

MOLECULAR CHARACTERISATION OF PFGE NON-TYPABLE METHICILLIN-RESISTANT STAPHYLOCOCCUS AUREUS IN THE NETHERLANDS, 2007

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In 2007 in the Netherlands, 30% of all human isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) sent to the National Institute for Public Health and the Environment could not be typed by pulsed-field gel electrophoresis (non-typable (NT)-MRSA). Molecular characterisation of the NT-MRSA isolates revealed 27 different spa types and two distinct SCCmec types, type IV and V. All NT-MRSA isolates were closely related based on spa and multi-locus sequence typing and belonged to the ST398 lineage. The rapid increase of NT-MRSA (ST398) isolates over the last years shows the importance of this relatively new clonal lineage.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an important pathogen causing not only infections in the hospital but also in the community. Lately, the epidemiology of MRSA is changing, focusing more and more on community-associated (CA)-MRSA. CA-MRSA are often Pantone-Valentine leukocidin (PVL)-

positive and can cause serious skin and soft-tissue infections, but also conditions such as necrotising pneumonia [1].

Since 2005, pigs have been identified as a possible new reservoir for MRSA [2,3]. Although the pig-related MRSA isolates are almost always PVL-negative, they could be considered as CA-MRSA. Pig-related MRSA strains can easily be identified by *Sma*I pulsed-field gel electrophoresis (PFGE). These strains possess a methylation enzyme that methylates the *Sma*I restriction sites. Consequently, no banding pattern is obtained in the PFGE and no PFGE type can be assigned [4]. Furthermore, staphylococcal protein A (*spa*) typing has shown that the pig-related strains belong to specific spa types, indicating a clonal structure. This clonality was also supported by multi-locus sequence typing (MLST), since the pig-related strains were all sequence type (ST) 398 or single locus variants of ST398 (ST752 and ST753) [5]. To date, the pig-related MRSA has also been found in other farm animals such as

TABLE 1

Incidence of spa types of PFGE NT-MRSA isolates in the Netherlands in 2007 (n=793)

Spa type	No. of isolates*	% of NT-MRSA*	% of all isolates*
t011	370	46.7	14.1
t108	268	33.8	10.2
t567	42	5.3	1.6
t034	20	2.5	0.8
t899	19	2.4	0.7
t571	15	1.9	0.6
t2330	11	1.4	0.4
t2123	8	1.0	0.3
t1456, t383	5 each	0.6	0.2
t1255, t3013	4 each	0.5	0.2
t588, t1184, t1457	3 each	0.4	0.1
t2582	2	0.3	0.1
t779, t943, t1451, t2287, t2329, t2748, t2971, t3014, t3053, t3146, t3208	1 each	0.1	0.0
Total	793	100	30.3

* Data per spa type

NT-MRSA: non-typable methicillin-resistant *Staphylococcus aureus*; PFGE: pulsed-field gel electrophoresis.

horses [6], poultry [7], and is associated with cattle [5]. Therefore, it is no longer considered a pig-related MRSA clone but a livestock-related clonal lineage.

In January 2003, the first MRSA was found in the Netherlands which could not be typed by PFGE. From then on it was referred to as PFGE non-typable (NT)-MRSA. In the following years, the number of NT-MRSA increased rapidly and due to the relation of NT-MRSA with pigs, several studies were performed in order to get an idea about the MRSA carriage rate of pigs and pig farmers [2,5,8-10]. Upon the first results it became clear that pigs could be considered as a reservoir for MRSA and pig-to-human transmission had occurred [11]. Although rarely seen to date, human infections due to ST398 MRSA have occurred [12-14]. Based on the results of the NT-MRSA studies, the Dutch national MRSA guidelines were adjusted in July 2006 and November 2007 to the effect that all individuals working or living in close contact with living pigs or cattle are isolated and screened for MRSA upon admission to a hospital. As a result of this new guideline the number of detected NT-MRSA isolates increased.

In the Netherlands, all MRSA isolates are sent to the national MRSA reference centre. The present paper gives an overview of NT-MRSA in the Netherlands in 2007, the molecular characteristics of NT-MRSA and the clonal structure of these isolates. The data will show the importance of this relatively new CA-MRSA clonal lineage.

Methods

Bacterial isolates

In the Netherlands, the National Institute for Public Health and the Environment (RIVM) serves as the national reference centre for surveillance of MRSA. All first MRSA isolates of newly identified carriers, one per patient, are sent to the RIVM for typing. All MRSA isolates submitted in 2007 were used for typing.

Typing

All MRSA isolates were typed by PFGE using *Sma*I as the restriction enzyme according to the HARMONY PFGE protocol [15]. The isolates that could not be typed by PFGE were selected and used for *spa* typing [16]. New *spa* types were assigned with Ridom Staphtype software version 1.5.13 (Ridom GmbH). For the analysis of *spa* types and the creation of a minimum spanning tree, Bionumerics software version 5.1 (Applied Maths) was used. The PVL genes were detected by PCR according to the method described by Lina *et al.* [11]. The first 300 NT-MRSA isolates of 2007 were used to determine the SCCmec type by multiplex PCR according to Kondo *et al.* [17]. In case the *spa* type of an isolate was different from those found among the first 300 NT-MRSA isolates, it was also subjected to SCCmec typing. Thus, a total of 308 isolates were analysed for SCCmec type. Furthermore, at least one isolate per *spa* type was subjected to MLST [18].

Results

In 2007, the RIVM received 2,619 unique MRSA isolates, of which 793 (30.3%) were non-typable by PFGE. *Spa* typing of these NT-MRSA isolates revealed 27 different *spa* types. Table 1 shows the incidence of the different *spa* types.

Two dominant *spa* types, t011 and t108, accounted for 80% of all NT-MRSA isolates and eleven *spa* types were only found once. In order to determine whether the 27 different *spa* types were related to each other, the repeats of each *spa* type were aligned (Table 2),

and a minimum spanning tree was made based on the *spa* types of the isolates (Figure).

All *spa* types were closely related, as confirmed by the MLST results. All isolates belonged to ST398. Two different SCCmec types were found among the 308 NT-MRSA that were tested: SCCmec type IV (n= 78) and type V (n=198) (Table 3).

For 32 isolates the SCCmec type could not be determined. For *spa* type t011, 139 NT-MRSA isolates were tested and were of SCCmec type IV (n=62) and type V (n=64), or could not be typed (n=13). Surprisingly, 98 isolates with *spa* type t108 were either SCCmec type V (n=93) or could not be typed (n=5). No SCCmec type IV was found among these 98.

Only one NT-MRSA isolate was PVL positive. This isolate had *spa* type t034 and the SCCmec PCR resulted in SCCmec type V. The PVL PCR was retested on this isolate and PVL was confirmed.

TABLE 2

Alignment of tandem repeats* of NT-MRSA *spa* types, Netherlands, 2007

spa	repeats													
t011	8	16	-	2	25	-	-	-	-	-	34	24	25	-
t034	8	16	-	2	25	-	2	25	-	-	34	24	25	-
t108	8	16	-	2	25	-	-	-	-	-	-	24	25	-
t567	8	-	-	2	25	-	-	-	-	-	-	24	25	-
t571	8	16	-	2	25	-	2	25	-	-	34	-	25	-
t588	8	16	-	2	-	-	-	-	-	-	-	24	25	-
t779	8	-	-	-	-	-	-	-	-	-	-	-	-	-
t899	7	16	23	2	-	-	-	-	-	-	34	-	-	-
t943	8	16	-	2	25	-	-	25	-	-	-	24	25	-
t1184	8	16	-	2	25	-	-	-	-	-	-	-	25	-
t1255	8	16	-	-	-	-	-	-	-	-	34	24	25	-
t1451	8	16	-	2	25	-	-	-	-	-	34	-	25	-
t1456	8	16	-	2	25	-	-	-	-	-	-	-	-	-
t1457	8	16	-	2	25	34	2	25	-	-	34	24	25	-
t2123	8	-	-	-	25	-	-	-	-	-	-	-	-	-
t2287	8	-	-	2	25	-	-	25	-	-	-	-	-	-
t2329	8	16	-	159	25	-	-	-	-	-	-	24	25	-
t2330	8	16	-	2	25	-	-	-	-	-	34	24	25	25
t2383	8	16	-	-	-	-	-	-	-	-	-	-	-	-
t2582	8	16	-	2	25	-	2	25	2	25	34	24	25	-
t2748	26	-	-	-	-	-	-	-	-	-	-	24	25	-
t2971	8	-	23	-	25	-	-	-	-	-	34	24	25	-
t3013	8	16	-	-	-	34	-	25	-	-	34	24	25	-
t3014	8	16	-	2	65	-	-	25	-	-	-	-	-	-
t3053	8	16	-	2	65	-	-	-	-	-	-	24	25	-
t3146	8	16	-	2	-	-	-	-	-	-	-	24	25	25
t3208	8	16	-	2	25	-	-	-	-	-	-	24	24	-

* Repeats with only one base difference are mentioned in the same column, e.g. repeat 7, 8, and 26. Repeat 16 and 23 also differ in one base but because they were also found together in *spa* type t899 they were put into a separate column.
NT-MRSA: non-typable methicillin-resistant *Staphylococcus aureus*.

Genetic relatedness of 793 NT-MRSA isolates, represented as a minimum spanning tree based on *spa* types

SCCmec type of PFGE NT-MRSA isolates in the Netherlands in 2007 (n=308)

NT-MRSA: non-typable methicillin-resistant *Staphylococcus aureus*; PFGE: pulsed-field gel electrophoresis.

Discussion

In the year 2003 the first MRSA was found in the Dutch national MRSA surveillance which could not be typed by PFGE. At that time the correlation between animals and NT-MRSA was unknown. All MRSA isolates in 2002 had been typable by PFGE. The importance of NT-MRSA became clear when an increasing number of these isolates were observed. After the MRSA screening guidelines were adjusted to include screening of all hospitalised patients who had close contact with living pigs or cattle, even more NT-MRSA isolates were recorded. In 2007, the NT-MRSA clone accounted for 30% of all MRSA isolates sent to the Dutch national reference centre (see Table 1). The MRSA isolates included in this study were those found through screening of patients in healthcare settings. NT-MRSA isolates found in research programmes were excluded.

Molecular characterisation of all NT-MRSA isolates from 2007 (n=793) showed 27 different *spa* types and two different *SCCmec* types. Two dominant *spa* types, t011 and t108, accounted for 80% of all NT-MRSA isolates. Eleven *spa* types were found only once. All *spa* types formed one clonal *spa* cluster (see Table 2 and Figure). MLST was performed on at least one isolate of each *spa* type. All isolates were of the ST398 lineage, indicating the clonal structure of these isolates.

The PVL prevalence of all MRSA isolates found in 2007 was 12% (data not shown). However, only one of the NT-MRSA isolates gave a product in a PCR for the PVL genes. Infection with the bacteriophage carrying the genes for PVL seems to be less common in the ST398 lineage. Nevertheless, several reports about infections [12-14] and one outbreak [19] caused by the ST398 lineage have recently been published. Furthermore, methicillin-susceptible ST398 *S. aureus* (MSSA) have been isolated from three human cases with bacteraemia [20], indicating that the ST398 MRSA lineage could pose a serious threat to public health if it retained the virulence of ST398 MSSA.

SCCmec typing of the NT-MRSA isolates revealed two *SCCmec* types, IV and V. These two *SCCmec* types are considered to be community-associated. Furthermore, 32 isolates could not be typed by *SCCmec*, indicating the emergence of one or more new *SCCmec* type(s). Remarkable is the difference between the two most prevalent *spa* types, t011 and t108. In MRSA isolates with *spa* type t011, both *SCCmec* types were found, whereas in isolates with *spa* type t108, only *SCCmec* type V was detected. It is conceivable that MRSA *spa* type t108 *SCCmec* type V may have originated from MRSA *spa* type t011 *SCCmec* type V by deletion of repeat 34. Since *SCCmec* is a mobile genetic element that carries the *mecA* gene, it is also possible that MRSA isolates could acquire different *SCCmec* types.

The increasing number of articles concerning the ST398 clone is an indication of the international emergence of this clone. In some European countries, as in the Netherlands, *spa* type t011 also dominates [21,22], while other countries reported other *spa* types as the dominant type [13,23,24]. Outside Europe, ST398 MRSA has also been found in the United States [25,26], Canada [9], Singapore [27], and China [28]. Generally, ST398 isolates of human and animal origin showed similar molecular characteristics, being non-typable by *Sma*I PFGE and having closely related *spa* types, and *SCCmec* type IV, V, or variants thereof.

Today, the ST398 MRSA lineage is no longer specifically seen in pigs, but has also been found in other animals such as horses [6], poultry [7], dogs [13], and associated with cattle [5]. A Dutch

study on the prevalence of MRSA in pigs identified *spa* types similar to the human *spa* types found in this study [2]. Currently, people in close contact with living pigs and cattle are screened upon admission to the hospital. Perhaps these guidelines will need to be adjusted again in the near future, since more animal species seem to serve as a reservoir for MRSA. Whether the number of NT-MRSA will increase even further remains to be seen. In the Netherlands, a country with a low prevalence of MRSA, transmission of MRSA within a clinical setting could be difficult to predict, as more than 80% of the NT-MRSA have either *spa* type t011 or t108.

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