Acta Alimentaria, Vol. 49 (3), pp. 307–313 (2020)
DOI: 10.1556/066.2020.49.3.9

MOLECULAR TYPING OF FOODBORNE COAGULASE-POSITIVE *STAPHYLOCOCCUS* ISOLATES IDENTIFIED BY MALDI-TOF MS

B. Horváth[a]*, F. Peles[b], A. Szél[c], R. Sipos[d], Á. Erős[d], E. Albert[f] and A. Micsinai[e]

[a]Institute of Food Science, Faculty of Agricultural and Food Sciences and Environmental Management, University of Debrecen Doctoral School of Animal Science, H-4032 Debrecen, Böszörményi út 138. Hungary
[b]Institute of Food Science, Faculty of Agricultural and Food Sciences and Environmental Management, University of Debrecen, H-4032 Debrecen, Böszörményi út 138. Hungary
[c]MikroMikoMed Ltd, H-1036 Budapest, Lajos utca 74–76. Hungary
[d]BIOMI Ltd, H-2100 Gödöllő, Szent-Györgyi Albert utca 4. Hungary
[e]WESSLING Hungary Ltd, H-1045 Budapest, Anonymus utca 6. Hungary
[f]University of Veterinary Medicine Budapest, H-2225, Üllő, Dóra major. Hungary

(Received: 12 February 2020; accepted: 12 April 2020)

The aim of the study was the identification and characterisation of coagulase-positive *Staphylococcus* bacteria obtained from food matrices by mass spectrometry and molecular methods. A total of 46 coagulase-positive *Staphylococcus* isolates were collected from different foodstuffs. The *Staphylococcus* isolates were identified by MALDI-TOF MS and confirmed by the presence and sequence analysis of the *Staphylococcus* protein A gene. Staphylococcal enterotoxin genes were also investigated by multiplex PCR. Based on the identification of strains by the MALDI-TOF MS technique and *spa*-typing, all strains were identified as *Staphylococcus aureus*. Based on their MS peak profiles, the isolates matched the spectra of three *S. aureus* reference strains in the Bruker MALDI Biotyper database, with identification scores higher than 1.999 in the case of all 46 (100%) isolates. The isolates showed great genetic variability. Twenty *spa* types were identified, from which most lineages are capable of colonizing humans. Fifty percent of the strains harboured at least one of four enterotoxin genes (*seg*, *seh*, *sei*, and *ser*), but none of the classical enterotoxin genes could be detected.

In the European Union, *Staphylococcus aureus* (*S. aureus*) is an important pathogenic agent of food intoxications (Denayer et al., 2017). *S. aureus* strains have several virulence factors such as lipases, thermonuclease, hyaluronidase, and haemolysins; however, the major virulence factors are the heat-stable enterotoxins that cause staphylococcal food poisoning (SFP) (Ortega et al., 2010). The onset of SFP symptoms is very rapid in contrast to other gastrointestinal infections, generally a short time after ingestion of the contaminated food (Mossong et al., 2015). In outbreaks, the symptoms typically include diarrhoea (89%), vomiting (87%), and abdominal cramps (72%); however, fever (9%) is rarely reported. Usually, pork (41% of pork dishes were ham) or poultry dishes (41% freshly prepared poultry dishes) are implicated (55%) in *S. aureus* outbreaks (Bennett et al., 2013).

Coagulase-positive *Staphylococcus* species have several defence mechanisms like immunoglobulin binding proteins – including staphylococcal protein A (SpA) – that assist the evasion of the host immune system (Foster, 2005). Protein A is produced by numerous species of *Staphylococcus*, and is a highly potent virulence as well as immune evasion factor (Balachandran et al., 2018). The 40–60 kDa cell-wall protein is secreted during the exponential growth phase and is encoded by the *spa* gene (Björk & Sjöquist, 1972). The synthesised protein is able to bind to the *Fc* and *F (ab')* 2 regions of immunoglobulins (Forsgren, 1970) and thus may interfere with the immunising ability of vaccines that depend on the production of opsonophagocytic antibodies, because these inhibit the functions of B-cells (Daum & Spellberg, 2012).

* To whom correspondence should be addressed.
E-mail: horvath.brigitta920108@gmail.com

The *spa*-typing of *S. aureus* isolates has two major aims: to analyse genetic microvariation in outbreak investigations and to analyse genetic macrovariation in population- and phylogenetic-based assaying (Hallin et al., 2009). A variable repeat region (*spa*) in the gene allows a reliable and rapid method to discriminate *S. aureus* isolates in outbreaks from those presumed to be epidemiologically unrelated (Koreen et al., 2004). Furthermore, the epidemiological definition makes it possible to compare the outbreaks occurring in clinical cases and animal husbandry. Given that *spa*-typing involves the sequencing of only one gene, it provides significant advantages in terms of time-to-result, standardisation, ease of use, and reproducibility as compared to other molecular biology techniques (Agius et al., 2007).

Matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS) allows the identification of microorganisms isolated from food or clinical cases by a low-cost, rapid, easy, high-throughput, and efficient identification technique (Croxatto et al., 2012). Using standardised and developed procedures, MALDI-TOF MS devices have revolutionised the identification at the species level of most Gram-positive and Gram-negative bacteria (Barnini et al., 2015). The present study aims to discriminate and perform molecular characterisation of foodborne coagulase-positive staphylococci typed molecular techniques after identification by MALDI-TOF MS.

1. Materials and methods

1.1. Isolates and culture conditions

The food samples were collected (January 2018–January 2019) in the WESSLING Hungary Ltd. Microbiological Laboratory. During the examination period, 46 coagulase-positive staphylococci were isolated by culturing on non-selective and selective growth media, based on the standard MSZ EN ISO 6888-1:2008. On Baird-Parker selective medium (Biokar, France), *Staphylococcus* isolates formed typical colonies. All strains were grown at 37 °C for 24±1 h on Columbia Blood agar (Neogen, UK), and all colonies showed a positive coagulase reaction. Samples were collected from different food matrices: poultry (n=8), beef (n=3), pork (n=20), venison (n=1), dried pasta (n=6), dairy products (n=3), ready meals (n=3), and vegetables (n=2).

1.2. Identification of Staphylococcus *spp. with MALDI-TOF MS*

For the identification, the colonies were grown on Columbia Blood agar. A formic acid suspension preparation protocol was used: a single colony was picked up with a sterile inoculation loop and suspended for 30 s in 40 µl of formic acid in a microtube. Then, 40 µl of acetonitrile was added to the suspension and mixed extensively. Finally, from the suspension, 1 µl was transferred onto the target plate; when dried, it was overlaid with 1 µl α-HCCA (10 mg ml^{-1}) matrix solution and left to dry.

Mass spectra were collected by application of a Bruker Microflex LT MALDI-TOF mass spectrometer operating in the molecular mass range of 2.0–25 kDa, in positive linear mode. *Staphylococcus* isolates were identified by MALDI BioTyper 3.1 software. More than 200 shots were required to give adequate spectra with an appropriate signal-to-noise ratio. Before each measurement, calibration was carried out with the Bruker Bacterial Test Standard using lyophilised *Escherichia coli.* During the measurement, 640 shots were performed with the aim of analysing the mass spectra of *Staphylococcus* spp.

The 46 isolates were analysed in parallel; the mass spectra obtained were compared with the Bruker MALDI Biotyper database, where mass spectra of six different *Staphylococcus aureus* isolates were available. The results are reported as numeric scores based on similarity with the reference spectra based on a proprietary algorithm of the Bruker MALDI Biotyper software comparing the presence and symmetry of peaks in the mass spectra of the unknown strain and the database strain entries. The software sets the following threshold score values for identification: scores between 2.300–3.000 are designated as "highly probable species identification", scores between 2.000–2.299 as "secure genus identification", scores of 1.700–1.999 as "probable genus identification", and scores below 1.699 are reported as "non-reliable genus identification". The closest matches are listed in order of score value, the highest indicating the highest similarity in the mass spectra.

1.3. Molecular analysis – spa-*typing*

The cheaper and less laborious *spa*-typing is a single-locus technique, which offers a subtyping resolution comparable to MLST and PFGE. During *spa*-typing, the sequence of a polymorphic VNTR (variable-number tandem repeat) sequence was investigated, which is located in the 3' coding region of the *S. aureus*-specific SpA. The *spa* type of a strain is determined by the repeat succession. There are exceptions; however, the special repeat length for the *spa* VNTR is usually 24 bp. The method used is based on the second version of the Protocol for PCR Amplification of spa Recommended by the EURL-AR (2012).

1.4. Staphylococcal enterotoxin (SE) gene PCR

Genomic DNA was obtained from *S. aureus* strains using PrepMan Ultra lysis buffer (Thermo Scientific, Biocenter Ltd, Szeged, Hungary) according to the manufacturer's protocol. The supernatant was diluted five-fold with TE buffer and used for PCR amplification. To detect the major SE genes (*sea*, *seb*, *sec*, *sed*, and *see*), multiplex PCR protocols were performed using the primer sets of the European Union Reference Laboratory for Coagulase-Positive Staphylococci (EURL-CPS) as published elsewhere (Bianchi et al., 2014) with a slight modification. Primers detecting the *seh* and *ser* genes were run as a third, separate reaction to avoid overlapping of the PCR products of similar size during the gel electrophoresis. Reference strains of *S. aureus* DSMZ 18586 (SEB), DSMZ 18587 (SEC, SEG, SEH, SEI), DSMZ 18588 (SED, SEG, SEI, SEJ, SER), DSMZ 18589 (SEE), and ATCC 29213 (SEA) were used as positive controls. Amplicons were separated by agarose gel electrophoresis and identified according to their predicted size.

2. Results and discussion

Table 1 includes the results of *spa*-typing and identification of strains by MALDI-TOF MS. Furthermore, the type of food and the best value and best match of identification are also listed, as are the enterotoxin genes harboured by the isolates. Most of the samples containing coagulase-positive staphylococci were collected from meat (70%) including pork (n=20), poultry (n=8), beef (n=3), and venison (n=1) and food made from them. As the total number and type distribution of sampled materials was not available, further conclusion on the isolation frequency of coagulase-positive staphylococci from different food matrices is not within the aims of this study.

Table 1. Identification of strains by MALDI-TOF MS and by *spa*-typing, and occurrence of staphylococcal enterotoxin (SE) genes

Category of food		ID number	Type of food sample	Best score	Organism (best match)*	*spa* type	SE genes typed detected
Dairy products		SA-1	Cheese	2.458	1	t091	*selp*
		SA-10	Cheese	2.440	1	t091	*selp*
		SA-18	Milky dessert	2.513	1	t084	–
Dried pasta		SA-2	Dried pasta	2.476	1	t127	*seh*
		SA-49	Dried pasta	2.308	1	t127	*seh*
		SA-7	Dried pasta	2.204	1	t084	–
		SA-21	Dried pasta	2.486	1	t084	–
		SA-22	Dried pasta	2.448	1	t084	–
		SA-11	Dried pasta	2.429	1	t084	–
Meat	Poultry	SA-12	Drumstick	2.465	1	t1491	*sei*
		SA-13	Drumstick	2.345	2	t002	*sei*
		SA-16	Marinated chicken	2.332	2	t3478	*sei*
		SA-44	Duck meat	2.401	1	t1491	–
		SA-17	Goose liver	2.447	1	t706	*sei*, *selp*
		SA-20	Duck meat	2.432	1	t1491	*seh*
		SA-28	Duck leg	2.353	3	t1491	*seh*
		SA-29	Duck greaves	2.571	1	t11807	–
	Pork	SA-4	Bacon	2.119	3	t963	–
		SA-31	Bacon	2.546	1	t7673	*seh*
		SA-5	Pork sausage	2.477	1	t1491	*seh*
		SA-6	Pork chops	2.166	1	t091	*selp*
		SA-32	Pork chops	2.466	1	t011	–
		SA-25	Pork sausage	2.429	1	Unknown	*seh*
		SA-33	Pork chops	2.329	1	t213	*selp*
		SA-24	Pork shoulder	2.243	1	t213	–
		SA-34	Pork chops	2.350	1	t1778	*seh*
		SA-9	Pork shoulder	2.425	1	t1778	*seh*
		SA-35	Pork shoulder	2.500	1	t1451	–
		SA-36	Pork shoulder	2.287	3	t213	*selp*
		SA-37	Pork shoulder	2.429	1	Unknown	–
		SA-15	Pork greaves	2.342	3	t091	*selp*
		SA-38	Pork sausage	2.474	1	t084	–
		SA-02	Pork shoulder	2.283	3	t15546	–
		SA-01	Pork sausage	2.295	2	t189	–
		SA-39	Pork knuckle	2.562	1	t084	–
		SA-40	Pork knuckle	2.516	1	t084	–
		SA-41	Pork cheek	2.403	2	t10349	*seh*
	Beef	SA-3	Beef	2.333	1	t091	–
		SA-8	Beef	2.484	3	t330	–
		SA-14	Beef offal	2.362	3	t084	–
	Venison	SA-42	Rabbit	2.371	1	t656	–
		SA-43	Burgers	2.282	3	t363	*sei*
Ready meals		SA-47	Grilled chicken	2.305	2	t1491	–
		SA-45	Chocolate	2.039	1	t330	*seg*, *sei*
Vegetables		SA-46	Fresh salad mix	2.525	1	t330	–
		SA-27	Carrot paste	2.421	1	t091	*selp*

* 1: *S. aureus* subsp. *aureus* DSM 799; 2: *S. aureus* subsp. *aureus* DSM 20231T; 3: *S. aureus* ATCC 33862 THL.

Based on the presence of the *spa* gene and on the best match and best score, all strains were identified as *S. aureus*. Based on the MALDI-TOF MS data, the isolates matched the spectra of three *S. aureus* reference strains in the Bruker MALDI Biotyper database. The number of the best-matching isolate is indicated in Table 1.

Using the formic acid suspension preparation method, it was possible to obtain identification scores higher than 1.999 in the case of all 46 (100%) isolates by comparison with the Bruker MALDI Biotyper database.The vast majority, 83% of the isolates, gave MS scores ≥2.300, so the species were reliably identified. Identification at the genus level was secure in 17% of isolates, and no isolates had a probable genus level identification with a score in the 1.700–1.999 range. Manukumar and Umesha (2017) obtained similar results when investigating foodstuffs: 34 out of 36 coagulase-positive *Staphylococcus* isolates were identified with a score >2.000, when using another method of direct sample preparation; however, no results of identification with a score over 2.300 were demonstrated.

The strains showed great genetic variability: 20 *spa* types were identified among the 46 isolates. The distribution of the *spa* types in food matrices is shown in Table 2. The most common *spa* type was t084 with an isolation frequency of 19.6% (9/46), belonging to human-related clonal complex (CC)15, and was found mostly in dried pasta and meat. Other frequent types were t091 (ST7) and t1491, both with an isolation frequency of 13.0% (6/46). These *spa* types are also frequently isolated from humans, and t1491 is associated with CC1. This lineage is widely distributed and consists of methicillin-susceptible and -resistant strains with low host specificity, thus frequently isolated from livestock and related products (Cuny et al., 2015).

Table 2. Distribution of *spa* types in food matrices

spa type (n=20)	Category of food								
	Dairy products (n=3)	Dried pasta (n=6)	Meat				Ready meals (n=3)	Vegetables (n=2)	Total number of isolates
			Poultry (n=8)	Venison (n=1)	Pork (n=20)	Beef (n=3)			
t002			1						1
t011					1				1
t084	1	4			3	1			9
t091	2				2	1		1	6
t127		2							2
t189					1				1
t213					3				3
t330						1	1	1	3
t363							1		1
t656				1					1
t706			1						1
t963					1				1
t1451					1				1
t1491			4		1		1		6
t1778					2				2
t3478			1						1
t7673					1				1
t10349					1				1
t11807			1						1
t15546					1				1
Unknown					2				2

The connection between the Bruker MALDI Biotyper database closest match reference strain and the *spa* type cannot be directly inferred: the 20 *spa* types were distributed among the three database reference strains, and several *spa* types were found to be assigned to several database entries (t084, t091, t213, t330, t1491).

Among the strains examined, 50% of the isolates contained at least one enterotoxin gene. In two strains (SA-17 and SA-45), two different types of SE were detected (Table 1). Four different enterotoxin genes were identified among the 46 isolates: *sei*, *seg*, *seh*, and *selp*, of which *seh* was the most prevalent at 17% (8/46) and which was found mostly in strains from pork meat. In SFP outbreaks, the predominance of SEA is well documented at 70–80% prevalence, independent of the region where the investigation has taken place (Argudín et al., 2010). Though other SEs and SE-like toxins (SE*l*), like SEH, may also be involved in clinical cases, their role in SFP is still controversial (Fisher et al., 2018). None of the major enterotoxin genes were present in our isolates, and only other SE genes of minor significance occurred in lower numbers, which may reflect favourable conditions in comparison with other studies on the matter (Li et al., 2015; Qi & Sangma, 2019).

3. Conclusions

Based on the results of *spa*-typing and the best match and best score with the MALDI-TOF MS technique, all strains were identified as *S. aureus*. The isolates matched the spectra of three *S. aureus* reference strains in the Bruker MALDI Biotyper database; 83% of the isolates gave scores ≥2.300, so the species were safely identified by mass spectrometry.

The MALDI-TOF MS technique is suitable for rapid and cost-effective identification of *S. aureus* among foodborne coagulase-positive *Staphylococcus* species in routine diagnostics. The correlation between the three MALDI Biotyper database *Staphylococcus aureus* reference strain entries and the *spa* types and enterotoxin genes could not be unambiguously established. This can be attributed to the fact that the six *Staphylococcus aureus* MALDI Biotyper database entries do not represent the genetic diversity within the species, and though it is enough for strain identification, but strain typing is not feasible due to such limitations.

Within the scope of the study, 4 different enterotoxin genes and 20 different *spa* types were identified among the 46 isolates. Around 50% of the isolates harboured enterotoxin genes (*sei*, *seg*, *seh*, and *selp*), but these are the SEs of lesser significance.

*

The study was supported by the EFOP-3.6.1-16-2016-00022 project, co-financed by the European Union and the European Social Fund. We would like to thank Dr. Judit Gasparik, Éva Róka, Ákos Pásztor, and Edit Pocklán for the support in the microbiology laboratory, and Dr. András Helenkár for his valuable insights on the MALDI TOF-MS technique.

References

Agius, P., Kreiswirth, B., Naidich, S. & Bennett, K. (2007): Typing *Staphylococcus aureus* using the *spa* gene and novel distance measures. *IEEE/ACM T. Comput.Bi.*, *4*, 693–704.

Argudín, M.Á., Mendoza, M.C. & Rodicio, M.R. (2010): Food poisoning and *Staphylococcus aureus* enterotoxins. *Toxins*, *2*(7), 1751–1773.

Balachandran, M., Bemis, D.A. & Kania, S.A. (2018): Expression and function of protein A in *Staphylococcus pseudintermedius*. *Virulence*, *9*, 390–401.

Barnini, S., Ghelardi, E., Brucculeri, V., Morici, P. & Lupetti, A. (2015): Rapid and reliable identification of Gram-negative bacteria and Gram-positive cocci by deposition of bacteria harvested from blood cultures onto the MALDI-TOF plate. *BMC Microbiol., 124*, 1–8.

Bennett, S.D., Walsh, K.A. & Gould, L.H. (2013): Foodborne disease outbreaks caused by *Bacillus cereus*, *Clostridium perfringens*, and *Staphylococcus aureus* – United States, 1998-2008. *Clin. Infect. Dis., 57*, 425–433.

Bianchi, D.M., Gallina, S., Bellio, A., Chiesa, F., Civera, T. & Decastelli, L. (2014): Enterotoxin gene profiles of *Staphylococcus aureus* isolated from milk and dairy products in Italy. *Lett. Appl. Microbiol.*, *58*(2), 190–196.

Björk, I.P. & Sjöquist, J. (1972): Some physicochemical properties of protein A from *Staphylococcus aureus*. *Eur. J. Biochem.*, *29*, 579–584.

Croxatto, A., Prod'hom, G. & Greub, G. (2012): Applications of MALDI-TOF mass spectrometry in clinical diagnostic microbiology. *FEMS Microbiol. Rev.*, *36*, 380–407.

Cuny, C., Wieler, L. & Witte, W. (2015): Livestock-associated MRSA: the impact on humans. *Antibiotics*, *4*(4), 521–543.

Daum, R.S. & Spellberg, B. (2012): Progress toward a *Staphylococcus aureus* vaccine. *Clin. Infect. Dis.*, *54*, 560–567.

Denayer, S., Delbrassinne,L., Nia, Y. & Botteldoorn, N. (2017): Food-borne outbreak investigation and molecular typing: high diversity of *Staphylococcus aureus* strains and importance of toxin detection. *Toxins*, *9*, 1–13.

European Union Reference Laboratory – Antimicrobial Resistance (EURL-AR) (2012): *Protocol for PCR Amplification of mecA, mecC (mecALGA251), spa and pvl Recommended by the EURL-AR, 2nd Version*. EURL-AR/National Food Institute, pp. 1–5.

Fisher, E.L., Otto, M. & Cheung, G.Y.C. (2018): Basis of virulence in enterotoxin-mediated staphylococcal food poisoning. *Front. Microbiol.*, *9*, 436.

Forsgren, A. (1970): Significance of protein A production by staphylococci. *Infect. Immun.*, *2*, 672–673.

Foster, T.J. (2005): Immune evasion by staphylococci. *Nat. Rev. Microbiol.*, *12*, 948–958.

Hallin, M., Friedrich, A.W. & Struelens, M.J. (2009): *spa* typing for epidemiological surveillance of *Staphylococcus aureus*. -in: Caugant, D. (Ed.) *Molecular epidemiology of microorganisms* (*Methods in Molecular Biology*, vol. 551). Humana Press, Totowa, NJ, pp. 189–202.

Koreen, L., Ramaswamy, S.V., Graviss, E.A., Naidich, S., Musser, J.M. &. Kreiswirth, B.N. (2004): spa typing method for discriminating among *Staphylococcus aureus* isolates: implications for use of a single marker to detect genetic micro- and macrovariation. *J. Clin. Microbiol.*, *42*, 792–799.

Li, G., Wu, S., Luo, W., Su, Y., Luan, Y. & Wang, X. (2015): *Staphylococcus aureus* ST6-t701 isolates from food-poisoning outbreaks (2006-2013) in Xi'an, China. *Foodborne Pathog. Dis.*, *12*, 203–206.

Manukumar, H.M. & Umesha, S. (2017): MALDI-TOF-MS based identification and molecular characterization of food associated methicillin-resistant *Staphylococcus aureus*. *Sci. Rep.*, *7*, 1–16.

Mossong, J., Decruyenaere, F., Moris, G., Ragimbeau, C., Olinger, C.M. …& Weicherding, P. (2015): Investigation of a staphylococcal food poisoning outbreak combining case–control, traditional typing and whole genome sequencing methods, Luxembourg, June 2014. *EuroSurveillance*, *20*, 30059.

MSZ EN ISO 6888-1:2008: *Élelmiszerek és takarmányok mikrobiológiája. Horizontális módszer a koagulázpozitív sztafilokokkuszok (*Staphylococcus aureus *és más fajok) számának meghatározására. 1. rész: Baird-Parker-agar táptalajos eljárás* (ISO 6888-1:1999). Microbiology of food and animal feeding stuffs. Horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species). Part 1: Technique using Baird-Parker agar medium (ISO 6888-1:1999).

Ortega, E., Abriouel, H., Lucas, R. & Gálvez, A. (2010): Multiple roles of *Staphylococcus aureus* enterotoxins: pathogenicity, superantigenic activity, and correlation to antibiotic resistance. *Toxins*, *8*, 2117–2131.

Qi, C. & Sangma, X. (2019): Genotypes, enterotoxin gene profiles, and antimicrobial resistance of *Staphylococcus aureus* associated with foodborne outbreaks in Hangzhou, China. *Toxins*, *11*(6), 307.