Agglutination of “*Streptococcus milleri*” by lectins

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Summary. The agglutination of 218 clinical isolates and three ATCC type strains of “*Streptococcus milleri*” was tested with 25 different lectins from plants and fungi. An agglutination reaction with one or more lectins was observed with 42 isolates when the cells were untreated. After trypsinisation of the bacteria, 109 strains yielded a positive reaction and after boiling the bacterial cells at pH 2, 218 isolates were agglutinated. As an overall result of our experiments with untreated, trypsinised and boiled cells, 17, 37 and 45 different agglutination patterns, respectively, were obtained. The lectins from *Datura stramonium*, *Robinia pseudoacacia* and *Dolichos biflorus* agglutinated isolates belonging only to Lancefield group C, being non-reactive with other isolates. These lectins were also found to be specific for “large colony type” streptococci of group C. The use of lectin agglutination in epidemiological and ecological studies of “*S. milleri*” is discussed.

Introduction

The species name “*Streptococcus milleri*” was first used in 1956 by Guthof to describe oral non-haemolytic streptococcal species.¹ Subsequently, the minute β -haemolytic and non-haemolytic streptococci referred to as the “*S. milleri* group” were included in this species.² This heterogeneous group of streptococci was separated into different species by Facklam.³ On the basis of biochemical and genetic similarities, Coykendall *et al.*⁴ proposed grouping of these streptococci in a single species, *S. anginosus*. However, by means of DNA–DNA hybridisation studies, Whiley *et al.* recently revealed that there are three DNA homology groups in strains classified as *S. anginosus*.⁵ Therefore, they proposed the re-instatement of three distinct species—*S. constellatus*, *S. intermedius* and *S. anginosus* in the nomenclature.⁶ However, the term “*Streptococcus milleri*”, although not included in the approved list of bacterial names,⁷ is used widely in clinical microbiology to describe streptococci that are found in suppurative infections and thus have a clinical significance.⁸

Strains belonging to the “*S. milleri*” group are heterogeneous: differences are found in many characteristics—including haemolysis,² fatty acid fingerprinting,⁹ DNA base-pair ratios¹⁰ and cell-wall sugar composition.¹¹ Furthermore, “*S. milleri*” strains can be differentiated serologically by their Lancefield group antigens (A, C, F or G), by Ottens type antigens (I, II, III or IV), or by both, but a significant number

of strains remains untypable.² Therefore, a typing system for these clinically important streptococci would be valuable for epidemiological and ecological studies.

In this report we describe the agglutination of 221 isolates of “*S. milleri*” by 25 different lectins from plants and fungi and discuss the usefulness of such a rapid, cheap and reliable typing method in epidemiological studies.

Materials and methods

Bacterial strains

In the period Jan. 1991–Dec. 1992, 218 clinical strains of “*S. milleri*” were isolated from 173 patients. Sites of isolation included the respiratory tract (40 strains), urogenital tract (24), blood (31), skin and soft tissues (35), abdominal cavity (26), pelvis (22) and other sites (40). Three type strains were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA): *S. anginosus* ATCC 33397, *S. constellatus* ATCC 27823 and *S. intermedius* ATCC 27335. The strains were stored at -70°C in peptone-glycerol (proteose peptone no. 2, 1% w/v; glycerin 8% v/v).

Lancefield grouping

Lancefield grouping of strains into groups A, B, C, F and G was performed with the streptex latex agglutination kit (Wellcome Diagnostics, Dartford) according to the manufacturer's instructions.

Table I. Lectins used in the agglutination assays

No.	Code	Species name	Common name	Carbohydrate specificity	Reference no.
1	PNA	<i>Arachis hypogaea</i>	Peanut	β -D-Gal(1,3)D-GalNAc	12
2	AMA	<i>Arum maculatum</i>	Lords and ladies	Complex	UP
3	AMA II	<i>Arum maculatum</i>	Lords and ladies	Complex	UP
4	APA	<i>Aegopodium podagraria</i>	Ground elder	D-GalNAc	13
5	ASA	<i>Allium sativum</i>	Garlic	α (1,3)Man	14
6	AUA	<i>Allium ursinum</i>	Ramsons	α (1,3)Man, α (1,6)Man	14
7	BDA	<i>Bryonia dioica</i>	White bryony	D-GalNAc	15
8	DSA	<i>Datura stramonium</i>	Thorn-apple	D-(GlcNAc) ₂	16
9	GNA	<i>Galanthus nivalis</i>	Snowdrop	α (1,3)Man	14
10	IRA	<i>Iris reticulatum</i>	Iris	D-GalNAc	UP
11	MAA	<i>Maackia amurensis</i>	Maackia	Neu5Ac- α (2,3)Gal	17
12	NPA	<i>Narcissus pseudonarcissus</i>	Daffodil	α (2,6)Man	14
13	RPA	<i>Robinia pseudoacacia</i>	Black locust	Complex	18
14	CCL	<i>Ceratobasidium cornigerum</i>	...	D-GalNAc	19
15	RSA	<i>Rhizoctonia solani</i>	...	D-GalNAc	19
16	SMyL	<i>Sclerotinia myabaena</i>	...	D-GalNAc	20
17	SSL	<i>Sclerotinia sclerotiorum</i>	...	D-GalNAc	20
18	SNA	<i>Sambucus nigra</i>	Elder	Neu5Ac- α (2,6)GalNAc	21
19	SNA II	<i>Sambucus nigra</i>	Elder	D-GalNAc	21
20	TL	<i>Tulipa</i>	Tulip	D-GalNAc	22
21	WFA	<i>Wisteria floribunda</i>	Wisteria	D-GalNAc	23
22	WGA	<i>Triticum aestivum</i>	Wheat germ	D-(GlcNAc), NeuNAc	24
23	Con-A	<i>Canavalia ensiformis</i>	Jack bean	α -D-Man, α -D-Glc	25
24	DBA	<i>Dolichos biflorus</i>	Horse gram	D-GalNAc	26
25	VAA	<i>Viscum album</i>	Mistletoe	β -D-Gal	27

UP, unpublished.

Lectins

Lectins from different plants and fungi were extracted, isolated by affinity chromatography and further purified as described previously.¹²⁻²⁷ The lectins are listed in table I, along with the scientific name and the common name of the species from which they were derived and their carbohydrate specificity. Lectin solutions of 1 mg/ml w/v in phosphate-buffered saline (PBS; 1.5 mM KH₂PO₄, 10 mM Na₂HPO₄, pH 7.4, 3 mM KCl, 140 mM NaCl) were stored at -20°C until use.

Agglutination assays

Bacterial strains were grown in 50 ml of Todd-Hewitt broth for 18 h at 37°C in air with CO₂ 5% v/v. Cells were collected by centrifugation at 3000 g for 15 min, washed in PBS and resuspended in PBS; suspensions were adjusted to an A₆₂₀ of 5 in a spectrophotometer.

Three ml of a suspension of each strain were left untreated, 3 ml were treated with trypsin and 3 ml were boiled in glycine-HCl. Trypsin treatment was with trypsin 0.1 mg/ml w/v for 1 h at 37°C. After incubation, the cells were washed twice in PBS to remove the trypsin and resuspended in 3 ml of PBS. For glycine-HCl treatment the cells were centrifuged at 3000 g for 15 min, resuspended in 3 ml of 0.25 M glycine-HCl buffer, pH 2.0, and boiled for 15 min. The cells were then washed with PBS and resuspended in 3 ml of PBS.

Agglutination assays were performed as previously

described²⁸ in small glass tubes in a final volume of 0.1 ml containing 10 μ l of lectin solution and 90 μ l of cell suspension. A negative control was included for each strain by adding 10 μ l of PBS instead of lectin solution.

Hapten inhibition test

To test whether the agglutination reaction of the lectin was caused by its carbohydrate-binding capacity, inhibition of agglutination was tested with several sugars. Ten μ l of a carbohydrate solution (1 M D-mannose, D-galactose, L-rhamnose, L-arabinose, N-acetyl-D-galactosamine, D-glucose, N-acetylglucosamine or D-fucose; or mucin from porcine stomach 1 mg/ml w/v) in PBS were added together with 10 μ l of the lectin solution to 80 μ l of cell suspension. After the mixtures had been left for 1 h at room temperature, agglutination was monitored visually.

Reproducibility

Reproducibility of lectin agglutination was tested with seven strains. Each strain was subcultured five times and the agglutination assays were performed as described above.

Results

Without pre-treatment of the bacteria, 42 strains agglutinated with one or more lectins (table II). The lectins PNA, APA, CCL, RSA and WFA gave the best

Table II. Agglutination of untreated cells of different Lancefield groups with different lectins

Lancefield group	Number of strains that agglutinated with each lectin*																	Total
	1	4	5	7	8	9	10	13	14	15	16	17	19	21	22	23	24	
A	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C	0	4	0	0	7	0	0	13	10	10	0	2	10	14	5	0	11	15
F	5	6	0	0	0	0	0	0	3	3	0	0	0	0	0	0	0	12
G	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1
NG	9	3	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	14
Total	14	14	0	0	7	0	0	13	14	14	0	2	10	14	5	3	11	42

NG, not groupable.

* Numbers correspond to those in table I.

Table III. Agglutination of trypsin-treated cells of different Lancefield groups with different lectins

Lancefield group	Number of strains that agglutinated with each lectin*																	Total
	1	4	5	7	8	9	10	13	14	15	16	17	19	21	22	23	24	
A	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C	0	20	0	0	26	0	0	27	27	27	0	14	26	27	27	0	27	27
F	12	28	0	0	0	0	0	0	10	10	1	1	0	1	6	4	0	33
G	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0	1	0	2
NG	25	21	0	1	0	0	0	0	12	15	3	5	9	0	7	13	0	47
Total	37	70	0	1	26	0	0	27	50	53	4	20	35	28	40	18	27	109

NG, not groupable.

* Numbers correspond to those in table I.

Table IV. Agglutination of boiled cells of different Lancefield groups with different lectins

Lancefield group	Number of strains that agglutinated with each lectin*																	Total
	1	4	5	7	8	9	10	13	14	15	16	17	19	21	22	23	24	
A	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1
C	0	16	0	0	28	0	0	31	31	31	0	15	27	31	33	0	31	33
F	11	70	12	0	0	6	0	0	33	36	0	0	0	1	82	1	0	82
G	0	8	1	0	0	0	0	0	2	5	0	0	0	1	9	0	0	9
NG	30	13	19	0	0	11	1	0	11	14	3	3	5	0	90	9	0	93
Total	41	107	32	0	28	17	1	31	77	86	3	18	32	33	215	10	31	218

NG, not groupable.

* Numbers correspond to those in table I.

results, each agglutinating 14 (33%) of the 42 strains. The *S. anginosus* type strain, belonging to Lancefield group G, agglutinated in the presence of APA, CCL and RSA while the other two (both ungroupable by Lancefield antisera) did not react with any lectin (table V). After trypsinisation of the bacteria, 109 strains, including the *S. intermedius* and the *S. anginosus* type strains, agglutinated in the presence of at least one of the lectins tested (tables III and V). APA gave the highest number, agglutinating 70 (64%) of the 109 strains (table III). When the bacteria were first boiled at pH 2, agglutination with one or more lectins was observed with 218 strains, including the three type strains (tables IV and V). WGA agglutinated 214 strains (98%), whereas PNA reacted with only 107 strains (49%) (table IV). The lectins AMA, AUA, MAA, NPA, SNA, TL and VAA did not agglutinate

any of the "*S. milleri*" strains tested, whereas lectins DSA, RPA and DBA reacted only with strains of Lancefield group C (tables II, III and IV).

The specificity of the agglutination was tested in a hapten inhibition test. Agglutination by all the lectins could be inhibited by their respective haptenic carbohydrates which are indicated in table I. Lectins DSA and RPA were also inhibited by D-GalNAc and mucin (results not shown).

Reproducibility testing revealed that lectin patterns were not altered after subculture of the bacteria. However, most strains displayed different agglutination patterns according to their pre-treatment. This phenomenon was also seen with the ATCC type strains (table V). Whereas *S. constellatus* reacted only when the bacteria were boiled, *S. intermedius* reacted after both boiling and trypsin treatment, but a different

Table V. Lectin agglutination of ATCC type strains after different treatments

ATCC strain	Treatment	Agglutination with lectins*																	
		1	4	5	7	8	9	10	13	14	15	16	17	19	21	22	23	24	
<i>S. constellatus</i> (No. 27823)	Untreated	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
	Trypsinised	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
	Boiled	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	—	—	
<i>S. intermedius</i> (No. 27335)	Untreated	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
	Trypsinised	+	+	—	—	—	—	—	—	+	—	—	—	—	—	—	—	—	
	Boiled	+	+	—	—	—	—	—	—	+	+	+	+	+	—	+	—	—	
<i>S. anginosus</i> (No. 22297)	Untreated	—	+	—	—	—	—	—	—	+	+	—	—	—	—	—	—	—	
	Trypsinised	—	+	—	—	—	—	—	—	+	+	—	—	—	—	—	—	—	
	Boiled	—	+	—	—	—	—	—	—	—	+	—	—	—	—	+	—	—	

* Numbers correspond to those in table I.

Table VI. Patterns of lectin agglutination most frequently obtained with untreated (U), trypsin-treated (T) or boiled (B) cells

Pattern no.	Agglutination with lectins*																		Number of strains		
	1	4	5	7	8	9	10	13	14	15	16	17	19	21	22	23	24	U	T	B	
1	—	+	—	—	+	—	—	+	+	+	—	+	+	+	+	—	+	2	10	9	
2	—	+	—	—	+	—	—	+	+	+	—	—	+	+	+	—	+	1	10	5	
3	—	—	—	—	+	—	—	+	+	+	—	—	+	+	+	—	+	2	2	8	
4	—	+	—	—	—	—	—	—	+	+	—	—	—	—	—	—	—	3	10	0	
5	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	13	8	0	
6	—	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	6	7	0	
7	+	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	10	0	
8	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	—	3	8	2	
9	+	+	—	—	—	—	—	—	—	—	—	—	—	—	+	—	—	0	2	10	
10	—	+	—	—	—	—	—	—	—	—	—	—	—	—	+	—	—	0	4	20	
11	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	—	—	0	4	40	
12	—	+	+	—	—	—	—	—	—	—	—	—	—	—	+	—	—	0	0	7	
13	—	+	—	—	—	—	—	—	+	+	—	—	—	—	+	—	—	0	0	31	
14	+	—	—	—	—	—	—	—	—	—	—	—	—	—	+	—	—	0	0	20	
15	—	—	+	—	—	—	—	—	—	—	—	—	—	—	+	—	—	0	0	9	

* Numbers correspond to those in table I.

Table VII. Discrimination given by lectin agglutination patterns obtained after different pre-treatments of the bacteria and in combination with Lancefield grouping among 221 isolates of "*S. milleri*"

Pre-treatment of the bacteria or combinations of pre-treatments	Without Lancefield grouping		With Lancefield grouping	
	Number of groups	Number of groups containing one isolate	Number of groups	Number of groups containing one isolate
Untreated	17	8	24	10
Trypsin-treated	37	19	48	25
Boiled at pH 2	45	22	58	28
Untreated + trypsin-treated	60	37	68	42
Untreated + boiled	72	46	87	48
Trypsin-treated + boiled	97	63	109	69
Untreated + trypsin-treated + boiled	118	90	130	97

agglutination pattern was obtained. After trypsinisation, this strain reacted with only three lectins, but after boiling at pH 2, it reacted with five more. The *S. anginosus* type strain gave the same agglutination pattern when trypsin-treated, as the untreated cells. However, after boiling the cells, only WGA gave a positive reaction.

When the bacteria were left untreated, 17 different agglutination patterns were obtained. Trypsinisation of the cells yielded 37 different patterns, 10 of which were identical to those obtained with untreated cells. Agglutination studies with bacterial cells boiled in 0.25 M glycine-HCl (pH 2) gave 45 different patterns, seven of which were identical to those obtained with

trypsin-treated bacteria and five to those obtained with both untreated and trypsin-treated bacteria. The 15 most frequently encountered lectin agglutination patterns are shown in table VI. The number of strains which agglutinated after each treatment are given for all the agglutination patterns. Patterns 1, 2 and 3 are specific for strains belonging to Lancefield group C. The other lectin patterns are not specific for one Lancefield group.

Only one strain did not react with any lectin untreated or after either treatment. One strain yielded the same agglutination pattern untreated and after both treatments, and 12, two and 19 strains, respectively, yielded the same pattern with untreated and trypsinised cells, with untreated and boiled cells, and with trypsinised and boiled cells.

Combining lectin agglutination with different pre-treatments of the bacteria markedly increased the discrimination between isolates (table VII). A combination of lectin agglutination patterns derived from untreated, trypsin-treated and boiled cells yielded 118 different groups, 90 of which contained only one isolate. The combination of lectin agglutination with Lancefield grouping yielded 130 different groups, 97 of which contained only one isolate, whereas Lancefield grouping alone gave only five groups (table VII).

Discussion

"*S. milleri*" is a heterogeneous group of bacteria differing in many bacteriological characteristics.⁸ Recently, an increasing number of these strains have been recognised as clinically important pathogens.²⁹ Therefore, a discriminatory, reproducible and rapid typing method for these bacteria would be helpful as an addition to Lancefield grouping, which distinguishes only four groups (A, C, F and G) and leaves almost 50% of the strains ungroupable. Moreover, most of the groupable strains belong to group F.⁶

In this study, the agglutination of 221 isolates of "*S. milleri*" by 25 different lectins was tested. The agglutination patterns were reproducible but differed according to the pre-treatment of the bacteria. With untreated, trypsin-treated and boiled isolates, 42, 109 and 218 isolates, respectively, reacted with at least one lectin, giving rise to 17, 37 and 45 different agglutination patterns. When the three agglutination patterns for each strain were considered, 118 different groups were found. In combination with Lancefield grouping of the bacteria, 130 groups were obtained. This indicates that the lectin agglutination assay is highly discriminatory and could be useful for epidemiological investigations. Since 97 of 130 groups contained only one isolate, it is clear that most bacterial isolates studied here possess unique surface structures. When the bacterial surface structures are partially destroyed by trypsin or by boiling in glycine-HCl at pH 2, some carbohydrate residues dissolve in the buffer while others, previously hidden, then become exposed on the

surface. Therefore, the agglutination patterns differed according to the pre-treatment of the bacteria. As agglutination by all the lectins could be inhibited by their respective haptenic carbohydrates, the agglutination reactions are specific lectin-carbohydrate bonds.

When the bacteria were first boiled at pH 2, WGA reacted with almost all strains, whereas without pre-treatment and after trypsinisation only five and 40 strains, respectively, agglutinated in the presence of WGA. This was also demonstrated with "large colony type" β -haemolytic streptococci.²⁸ Probably the teichoic acids and the peptidoglycans, which can bind to WGA,³⁰ become exposed on the bacterial surface only after boiling at pH 2.

Some of the patterns were specific for strains of Lancefield group C. These patterns are the same as those described earlier among other β -haemolytic streptococci.²⁸ Indeed, the lectins from *Datura stramonium* (DSA), *Robinia pseudoacacia* (RPA) and *Dolichos biflorus* (BDA) agglutinate specifically "*S. milleri*" isolates of group C as well as those strains belonging to the "large colony type" β -haemolytic streptococci of group C.²⁸ However, the lectins WFA, CCL, SSL and SNA II, described as agglutinating specifically β -haemolytic group C streptococci,^{28, 31} also reacted with "*S. milleri*" isolates of other Lancefield groups. The same phenomenon has been observed in studies of the *Helix pomatia* lectin with "*S. milleri*" and led to the assumption that antigens other than the group-specific C-polysaccharide may be involved in the recognition and binding of the lectin.³² Indeed, the "minor antigens" of Ottens and Winkler have been observed in "*S. milleri*".³³ The group-specific polysaccharide of Lancefield group C streptococci is a terminal α -N-acetyl-D-galactosamine coupled to the poly-L-rhamnose backbone.³⁴ Despite the facts that DSA shows specificity towards GlcNAc and that RPA has a complex specificity, they both agglutinate only group C streptococci, and both are able to bind to the group-specific antigen as the agglutination of both lectins can be inhibited by 0.1 M D-GalNAc.

It became clear from the agglutination assays with diverse lectins that, despite their different ecological and pathogenic characteristics, the "minute colony"-forming bacteria of the "*S. milleri*" group and the "large colony"-forming streptococci have the same cell-wall structures. Nevertheless, the cell wall is an important virulence factor in many bacteria. It is possible that the cell walls differ in components other than those reacting with the lectins, so these differences cannot be detected by lectin agglutination assays.

Lectin agglutination is cheap, rapid and easy to perform and is highly discriminatory; the results are reproducible—all of which makes it suitable for epidemiological and ecological studies of "*S. milleri*". However, for practical purposes, it is advisable to use only boiled cells and an agglutination assay with only six lectins. When the lectins PNA, APA, GNA, RSA,

Con-A and DBA are used, 76% of strains can be divided in 18 groups with different lectin patterns.

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