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264 Enhanced Interleukin-1β Release and Longevity
of Glioma-associated Peripheral Blood Monocytes in
Vitro
Experimental Study

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ABSTRACT: INTERLEUKIN-1 (IL-1) PLAYS A
controversial role in the immune response. Besides its
activation of immune cells and juvenile central
nervous system cells, monocyte-derived IL-1 may be
able to stimulate the malignant transformation and
proliferation of glial brain tumor cells expressing IL-1
receptors. The aim of this study was to determine
the growth pattern and the IL-1β release of long-term
cultured peripheral blood monocytes of glioma
patients. At 6- to 7-day intervals, the vital monocytes,
characterized by CD14 immunophenotyping, were
counted. By the use of a specific IL-1β enzyme-
linked immunosorbsent assay, the IL-1β content of
monocyte culture supernatants derived from 13
subjects with glioma and from 12 controls were
compared at Days 7, 21, and 100 of culture. Cell
clusters of monocytes derived from glioblastoma
patients survived more than 250 days in culture,
whereas control monocytes survived only up to 114
days. The IL-1β release of glioma-associated
peripheral blood monocyte cultures was about 50
times higher as compared with control monocyte
cultures. Dexamethasone treatment at the time of
blood sampling and recurrences of the gliomas did
not influence the increase in the IL-1β expression of
glioma monocytes. It seemed that at least subsets of
glioma-associated blood monocytes, although they
had been removed from the circulation, remained
activated for a long period of time. We conclude that
increased IL-1β production of glioma-associated
peripheral blood monocytes and their longevity in
vitro may be features of aberrant immune cell
subsets. In future studies, the exact phenotyping of
monocyte subsets will be mandatory.

KEY WORDS: Cytokine; Glioma;
Immunosuppression; Interleukin-1β; Macrophage;
Monocyte

In physiological and pathological conditions,
peripheral blood monocytes are known to leave the
cerebral circulation and become brain tissue
macrophages, i.e., microglia. In addition, there is evidence that microglial cells may
enter the circulation and thus become peripheral
blood monocytes again.

Monocytes/macrophages play a central role in the
glioma-associated immune response because of their
capability of tumor antigen incorporation, antigen
processing, and antigen presentation to T
lymphocytes. After contact with macrophage
membrane-bound tumor antigen, T lymphocytes only
proliferate and lyse tumor cells if they get activated
by interleukin-1 (IL-1). This cytokine exists in two
forms, membrane-bound IL-1α and free IL-1β, which
is released into the extracellular space. IL-1β
activates circulating monocytes and influences their
apoptotic cell death. IL-1 and its receptors are
produced by cells of both the immune system
(macrophages, microglia) and the
nervous system (neurons, astrocytes). The
receptors for IL-1 (IL-1R) also exist in at least two
forms. One form of the IL-1R is present on T cells,
monocytes, macrophages, and microglia.

Recent studies have provided strong
evidence of IL-1 release and of IL-1R expression not
only in immune cells and normal central nervous
system cells but also in glioma cells. Thus, IL-1 plays
a controversial role in the glioma-associated immune
response. On the one hand, this cytokine is necessary
to induce the activation and proliferation of immune
cells and juvenile central nervous system cells; on the
other hand, it is able to stimulate the proliferation of
brain tumor cells. For instance, it was reported that IL-
1 has mitogenic properties in fibroblasts and
astrocytes because these cells possess at least one
form of IL-1R. Giulian and Lachman demonstrated that IL-1 stimulated astroglial
proliferation after brain injury. Bertoglia et al. found that IL-1 derived from a B cell line promoted
the proliferation of an astrocytoma cell line in vitro.
Taken together, these studies indicate that glioma
cells and tumor-associated monocytes/macrophages
may stimulate each other via IL-1β in an autocrine or
paracrine manner. The aim of this study was to
determine the growth pattern and the IL-1β release of
cultured monocytes of patients with primary and
recurrent glioblastoma multiforme.

PATIENTS AND METHODS
Composition of the study groups
The peripheral blood monocytes of 14 patients who
underwent open or stereotactic surgery for primary
glioblastoma multiforme (n = 7) and recurrent
glioblastoma (n = 7) were investigated (Table 1). The
grading of the tumors was based on histological
criteria according to the World Health Organization
classification of brain tumors.

One of seven patients with a primary manifestation
of the tumor was on oral dexamethasone (12 mg/d).
Six of seven patients with recurrent glioblastoma
multiforme were on oral dexamethasone (dosage
ranging from 3 to 12 mg/d). Six of seven patients
with a primary manifestation of the tumor had not
received any dexamethasone at the time of blood
sampling.

Blood samples were taken by peripheral venous
puncture at 1 to 4 days before surgery. The peripheral
blood monocytes of five patients who had suffered
subarachnoid hemorrhage, cerebro-vascular spasm, and
subsequent brain infarction as well as monocytes from seven healthy donors formed the control group (Table 1).

Separation of monocytes
Twenty-four-milliliter samples of citrated venous blood were mixed gently with the same aliquot of Hanks’ Balanced Salt Solution (HBSS) (Sigma, Deisenhofen, Germany). This mixture underwent density gradient centrifugation (room temperature, 15 min, 300g) over Ficoll (Biochrom KG, Berlin, Germany). After centrifugation, the buffy coats containing lymphocytes, monocytes, and platelets were collected and washed twice in 10 ml of HBSS (centrifugation for 10 min at 100g each) to separate mononuclear cells from contaminating platelets. Cell viability was over 98% as assessed by trypan blue incorporation.

At the same time, differential white blood cell counts were performed. Blood samples were mounted on glass coverslips, air dried, and stained with May-Grünwald and Giemsa stains. The mean values of lymphocyte and monocyte numbers, determined by the counting of at least 600 white blood cells by two different investigators, served to represent the ratio of lymphocytes to monocytes in the peripheral venous blood of each subject. This ratio was used to calculate the number of monocytes seeded into cell culture dishes (35 and 100 mm in diameter; Falcon, Becton Dickinson GmbH, Heidelberg, Germany).

One hundred thousand monocytes per 35-mm dish and 5 x 10^5 monocytes per 100-mm dish were cultured in RPMI 1640 (Sigma) supplemented with 20 mmol of glutamine, penicillin, streptomycin, and 5% fetal calf serum (endotoxin content, less than 2.5 U/ml; Gibco/BRL Life Technologies, Eggenstein, Germany). Multiple 35-mm dishes were used to compensate for any accidental loss of 100-mm dishes, in which the experiments were carried out. Incubation was at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. After 48 hours, the dishes were washed twice with HBSS to remove nonadherent lymphocytes. The adherent monocytes were used for the in vitro experiments described in this study.

Culture conditions
The monocyte cultures were kept in an incubator (Nunc, Wiesbaden, Germany) under constant conditions (37°C, 5% CO₂) in a humidified atmosphere. At every 7th day, the monocyte cultures were washed twice with HBSS to remove cell debris and nonadherent dead cells. The cell death of the decanted mononuclear cells was proved by trypan blue incorporation. After the washing procedure, each culture received 2 ml (35-mm dishes) or 10 ml (100-mm dishes) of fresh culture medium.

Determination of cell numbers and of cell culture survival time
At 6- to 7-day intervals, the monocyte culture dishes of 14 patients with glioblastoma and of 12 controls were screened for adherent vital cells. The number of surviving monocytes in each culture dish was assessed by the counting of the adherent cells within five randomized optical fields of a phase contrast inversion microscope (Leitz, Wetzlar, Germany) at a magnification x100.

Each culture was brought to a termination when cell numbers had reduced to fewer than 10 in each of the 5 optical fields. By this time, the culture was defined as avital. Kaplan-Meier curves (19) were calculated from the survival periods of culture dishes derived from 7 patients with primary glioblastoma, 7 patients with recurrent glioblastoma, and 12 controls.

Determination of cell type and Interleukin-1β release
Culture supernatants were obtained before the washing procedure at Days 7 and 21 and, if cells were still vital, at Day 100. After centrifugation (room temperature, 5 min, 400g), the material was immediately frozen and stored at -80°C. Randomized samples of culture supernatants of each subject were quantitatively analyzed for soluble CD14 (sCD14), a surface antigen restricted to monocytes and immature macrophages. Analysis was performed with a commercially available specific enzyme-linked immunosorbent assay (ELISA) detecting sCD14 (IBL, Hamburg, Germany).

In addition, the immunophenotype of cultured cells was determined by immunofluorescence staining at randomized intervals. Cells were incubated in the dark with fluorescein isothiocyanate-conjugated mouse antihuman Leu-M3 monoclonal antibodies at saturating amounts at 4°C for 20 minutes and were subsequently washed with ice-cold phosphate-buffered saline. The Leu-M3 monoclonal antibodies (Becton-Dickinson, Heidelberg, Germany) were directed against the monocyte/macrophage phenotype antigen CD14. Microphotographs were taken in a semidark chamber with a fluorescence microscope (Zeiss, Oberkochen, Germany) and Kodak Ektachrome 64 Professional Tungsten films (Eastman Kodak, Rochester, NY). The C6 rat glioma cell line served as a nonmonocyte control cell line.

Additionally, the culture supernatants were analyzed for the cytokine IL-1β at Days 7, 21, and 100. A commercially available IL-1β-specific ELISA (Hermann Biemann GmbH Diagnostica, Bad Nauheim, Germany) was used.

Statistical analysis
The cell numbers in each culture dish were used to calculate the concentration of sCD14 and IL-1β per 10⁵ monocytes. Mean values and standard errors of sCD14 and IL-1β release per 10⁵ cells each were calculated for the following experimental groups: all glioblastoma, primary glioblastoma, recurrent glioblastoma, glioblastoma without dexamethasone therapy, glioblastoma with dexamethasone therapy, and controls. Differences between the groups were evaluated by use of the Kruskal-Wallis test with multiple comparisons on ranks of several independent samples (30)

Survival curves were drawn by the use of the Kaplan-Meier product-limit method (19). The log-rank or Mantel-Haenszel test was applied to evaluate
differences between the survival curves.

RESULTS

Survival of cultured monocytes
Survival data for all monocyte cultures are shown in Figure 1. All Kaplan-Meier curves of survival probability had an almost sigmoidal shape, with a shift to the right of the curves derived from glioblastoma-associated monocytes, indicating a delay of cell death in the latter. Typically, in those cultures, clusters of macrophages remained viable for long periods, a feature never observed in controls.

The median survival for control cultures was 61 days, and, for glioma-associated cultures, it was 100 and 107 days (primary and recurrent glioma, respectively). By Day 114, all control monocyte cultures had died, whereas 14.3% of monocyte cell cultures derived from glioblastoma patients survived for more than 250 days. This difference was significant ($P < 0.001$, log-rank test). Even at Day 250 of culture, when the culture experiments for this study were terminated, some glioma-associated monocytes depicted protruding growth cones, active migration, and cell-to-cell contacts with neighboring monocytes (Fig. 2).

CD14 monocyte surface antigen expression
The CD14 monocyte phenotype marker, as determined by immunostaining and with a specific sCD14 ELISA in the supernatants of cultures, did not change throughout the time of observation both in cultures of glioma patients and in those of control subjects (Fig. 3). This demonstrates that cultures derived both from glioma patients and from controls mainly retained cells of the monocyte phenotype. At Day 7, the supernatants of glioblastoma monocyte cultures contained $0.55 \pm 0.04$ (mean $\pm$ standard error) ng of sCD14/10^3 monocytes and those of control cultures contained $0.38 \pm 0.04$ ng of sCD14/10^3 monocytes. At Day 21, the sCD14 content of supernatants was $0.52 \pm 0.06$ ng/10^3 monocytes derived from tumor patients and $0.51 \pm 0.08$ ng/10^3 monocytes derived from control subjects. At Day 100, the CD14 content of culture supernatants was $0.48 \pm 0.03$ ng/10^3 monocytes derived from tumor patients. At Day 100, only one control culture retained vital cells left that had not undergone cell death, and the sCD14 content of this control supernatant was below the level of detection. In contrast, at this time, 57% of glioma monocyte cultures contained many cells growing in cluster-like formations.

There were no significant differences of sCD14 content between monocyte cultures derived from patients with primary tumors and those with recurrent glioblastomas. The dexamethasone treatment of glioblastoma patients at the time of blood sampling also did not significantly influence the IL-1β content of monocyte culture supernatants (Fig. 4). At Day 7, the IL-1β content of glioblastoma monocyte culture supernatants was $1.01 \pm 0.11$ (mean $\pm$ standard error) pg/10^3 cells as compared with $0.21 \pm 0.06$ pg/10^3 control monocytes. This difference was significant ($P \leq 0.01$). At Day 21, the IL-1β content of supernatants increased to $5.02 \pm 0.60$ pg/10^3 monocytes derived from tumor patients and decreased to only $0.10 \pm 0.04$ pg/10^3 monocytes derived from control subjects. Statistical analysis showed a significant difference ($P \leq 0.001$) of IL-1β content between both groups at Day 21. At Day 100, the IL-1β content of culture supernatants was $3.31 \pm 0.38$ pg/10^3 monocytes derived from brain tumor patients. At Day 100, the IL-1β content of one control supernatant was below the level of detection.

There was no significant difference of IL-1β content between monocyte cultures derived from patients with primary tumors and those with recurrent glioblastoma (Fig. 5). At Day 7, the IL-1β content was $1.13 \pm 0.38$ pg/10^3 monocytes derived from subjects with primary lesions and $0.91 \pm 0.04$ pg/10^3 monocytes from patients with recurrent glioblastoma. At Day 21, the IL-1β content increased to $5.19 \pm 1.48$ and $4.85 \pm 1.01$ pg/10^3 monocytes, respectively. At Day 100, the IL-1β content of primary glioblastoma monocyte cultures was $3.29 \pm 1.65$ pg/10^3 cells, and the IL-1β content of recurrent glioblastoma monocyte cultures was $3.34 \pm 0.55$ pg/10^3 cells.

The dexamethasone treatment of glioblastoma patients at the time of blood sampling also did not significantly influence the IL-1β content of monocyte cultures (Fig. 6). At Day 7, the IL-1β content of culture supernatants was $0.82 \pm 0.12$ pg/10^3 monocytes derived from patients treated with dexamethasone and $1.24 \pm 0.46$ pg/10^3 monocytes from brain tumor patients without dexamethasone treatment. At Day 21 of culture, the IL-1β content was $4.38 \pm 1.34$ pg/10^3 monocytes from subjects with dexamethasone and $5.66 \pm 1.78$ pg/10^3 monocytes from subjects without dexamethasone. At Day 100, the IL-1β content of monocyte culture supernatants was $3.26 \pm 0.96$ pg/10^3 cells from glioblastoma patients with dexamethasone and $3.37 \pm 1.33$ pg/10^3 mononcytic cells from glioblastoma patients without dexamethasone.

DISCUSSION

Tumor cells and tumor-associated monocytes release Interleukin-1β
At Day 21, the supernatants of cultured peripheral blood monocytes of glioblastoma patients contained about 50 times more IL-1β than did those of healthy donors as well as those of patients with subarachnoid hemorrhage, vasospasm, and brain infarction. Moreover, the IL-1β content of culture supernatants was not influenced by the dexamethasone treatment of patients at the time of blood sampling. In addition, the IL-1β production of cultured monocytes did not differ between cells derived from subjects with primary glioma and those with recurrent tumors.

Because it is well known that IL-1β has a central function in the autoregulatory circuits of immune cell proliferation, the 50-fold increase in IL-1β content in
culture supernatants and the pronounced longevity of subpopulations of glioma-associated monocytes in this study may indicate an imbalance of immune cell regulation leading to aberrant macrophages in glioma patients. Recently, it has been shown that malignant tumors not only induce an increase in IL-1\(\beta\) production by monocytes/macrophages, but also that glioblastoma cells in vitro and in vivo synthesize messenger ribonucleic acid encoding for IL-1\(\beta\) and for IL-6 \(^{21}\) and express IL-1\(\beta\), IL-6, transforming growth factor-\(\beta\), and tumor necrosis factor proteins \(^{4,9}\). Similar findings were provided by Evans et al. \(^{7}\), who reported that progressing murine sarcomas not only produce IL-1 but also may induce high levels of IL-1 systemically in association with macrophages. In addition, those authors \(^{7}\) found progressively growing tumors to release other biologically active molecules into the circulation that induce IL-1\(\alpha\), IL-1\(\beta\), tumor necrosis factor-\(\alpha\), and IL-6 gene expression in macrophages. One could speculate whether substances produced by glioma cells are able to induce and sustain IL-1\(\beta\) gene expression in glioma-associated monocyte subsets as well \(^{15}\).

**Autocrine/paracrine stimulation of monocytes by Interleukin-1\(\beta\)**

The increased IL-1\(\beta\) production of nonstimulated, long-term cultured blood monocytes of glioblastoma patients in our study could be a feature of selectively activated monocyte subsets. This finding is not contradictory to data demonstrating that short-term cultured glioma-associated monocytes produce IL-1 levels comparable with those released by monocytes from healthy persons when activated by lipopolysaccharides \(^{8}\). The synthesis of our data and those presented by Elliott et al. \(^{6}\) corroborates the assumption that glioblastoma-associated circulating monocytes are maximally activated cells. This kind of activation seems to be related to the inflammatory response against the tumor, because such activated cells could not be found in cultures derived from control subjects with brain infarction, although quite frequently, in pathological studies, an inflammatory border zone around infarcted brain tissue can be seen.

It seems possible that the pronounced delay of monocyte cell death observed in our experiments at least partially was caused by an autocrine or paracrine stimulation via IL-1\(\beta\). Because monocytes are known to express IL-1R \(^{2,18,23}\), an autocrine stimulation would lead to evolutionary advantages of monocytes with a high IL-1\(\beta\) production. Such autocrine or paracrine stimulation may be the reason that about 14% of glioblastoma-associated monocyte cell cultures survived for more than 250 days after having been removed from the circulation and from any effect of substances shed by the tumor. Therefore, the longevity as well as the increase in the IL-1\(\beta\) production of glioma-associated monocytes from Days 7 to 21 of culture might be due to a selective loss of suppressor monocytes in these cultures. The significance of such monocyte subsets in vivo may be their ability to stimulate the growth of glioma cells carrying IL-1\(\beta\) receptors, provided that these monocytes infiltrate into the tumors. If they do, the enhanced IL-1\(\beta\) release of such monocytes may be part of an explanation for the not entirely consistent findings showing malignant tumors, such as glioblastoma multiforme, on the one hand retaining more tumor-infiltrating macrophages than benign lesions \(^{4,6,14,26,28}\) and, on the other hand, inducing immunosuppression \(^{1,5,6,15,24-27,29}\). Hence, in future studies, it will be valuable to characterize the peripheral blood monocytes and the cerebral tissue macrophages of glioma patients.

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COMMENTS
Using tissue culture flasks, the authors have observed a differential in the survival of monocytes derived from the peripheral blood of glioblastoma patients versus control patients. At the same time, the former cells were shown to produce much greater quantities of interleukin-1.

The observation that the monocytes derived from glioblastoma patients produce greater quantities of interleukin-1 than do control monocytes is not surprising, considering the immunocompromised status of glioblastoma patients. It is, however, surprising to see that the altered expression of interleukin-1 by monocytes is maintained in vitro for such a long period of time; this indicates, most likely, an alteration of the monocytes at the genome level. It will be critical in future studies to confirm...
this hypothesis and especially to elucidate the precise mechanisms through which the brain tumor can affect the genetic expression of peripheral blood cells.

Raymond Sawaya
Houston, Texas

The role of interleukin-1β in the growth of a variety of neoplasms has been of significant interest. This is one of a series of cytokines that appears to have multiple roles in directly and indirectly affecting cells of the immune system as well as tumor cells.

In this study, interleukin-1β production has been found to be increased in the peripheral blood monocytes of glioma patients. The relationship between the production of this cytokine interleukin-1β and glioma growth represents an intriguing issue in understanding the role of cytokines in glioma patients.

Paul L. Kornblith
Pittsburgh, Pennsylvania
Figure 1. Graph showing survival data of peripheral blood monocyte cultures of 12 nontumor controls and of 14 patients with glioblastoma (7 primary, 7 recurrent) by the use of the Kaplan-Meier method (19). Note the almost sigmoidal shape of the survival curves and the shift to the right of curves derived from glioblastoma-associated monocytes, indicating delay of in vitro cell death of these monocytes. The difference in survival periods between controls and glioma-associated monocytes was significant ($P < 0.001$, log-rank test).
Figure 2. Photomicrograph (original magnification \( \times400 \)) of long-term cultured peripheral blood monocytes derived from a 61-year-old woman with glioblastoma (Tumor Patient 9 in Table 1). Subpopulations of these monocytes did not undergo cell death until Day 250 of culture, when the experiments of this study were terminated. Note the protruding growth cone (arrow), indicating migration, and the contact of neighboring monocytes (triangle).
Figure 3. Graph showing sCD14 monocyte/macrophage lineage marker content per $10^3$ cells at Days 7, 21, and 100 in culture supernatants of peripheral blood monocytes. The monocytes were derived from control subjects and from glioblastoma patients. There were no significant differences between both groups, and there was no significant change of sCD14 content throughout the time of culture. This demonstrates that the cultured cells of both groups were white blood cells of the monocyte/macrophage lineage.
Figure 4. Graph showing the IL-1β content per 10^3 cells at Days 7, 21, and 100 in culture supernatants of peripheral blood monocytes. The monocytes were derived from control subjects and from glioblastoma patients. At Day 7, the IL-1β content of glioblastoma monocyte culture supernatants was about 5 times higher (*P ≤ 0.01, Kruskal-Wallis test) and at Day 21 was about 50 times higher (**P ≤ 0.001) as compared with control cultures.
Figure 5. Graph showing the IL-1β content per $10^3$ cells at Days 7, 21, and 100 in culture supernatants of peripheral blood monocytes. The monocytes were derived from subjects with primary glioblastoma and recurrences. Statistical analysis revealed the differences between both groups to be not significant.
Figure 6. Graph showing the IL-1β content per $10^3$ cells at Days 7, 21, and 100 in culture supernatants of peripheral blood monocytes. The monocytes were derived from glioblastoma patients with dexamethasone (dex) treatment and without dexamethasone at the time of blood sampling. There were no differences between the two groups.
Table 1. Composition of the Study Groups

| No. | Healthy donor | Healthy donor | Healthy donor | Healthy donor | Healthy donor | Healthy donor | Healthy donor | Healthy donor | Healthy donor | Healthy donor | SHV anamn., VSS | SHV anamn., VSS | SHV anamn., VSS | SHV anamn., VSS | SHV anamn., VSS | SHV anamn., VSS | Healthy donor | Healthy donor | Healthy donor | Healthy donor | SHV anamn., VSS | SHV anamn., VSS | SHV anamn., VSS | SHV anamn., VSS | Number of patients |
|-----|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|----------------|----------------|----------------|----------------|----------------|----------------|---------------|---------------|---------------|---------------|----------------|----------------|----------------|----------------|----------------|
| 1   | Yes           | No            | No            | No            | No            | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes            | Yes            | Yes            | Yes            | Yes            | Yes            | Yes           | Yes           | Yes           | Yes           | Yes            | Yes            | Yes            | Yes            | Yes            | 2             |
| 2   | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes            | Yes            | Yes            | Yes            | Yes            | Yes            | Yes           | Yes           | Yes           | Yes           | Yes            | Yes            | Yes            | Yes            | Yes            | 2             |
| 3   | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes            | Yes            | Yes            | Yes            | Yes            | Yes            | Yes           | Yes           | Yes           | Yes           | Yes            | Yes            | Yes            | Yes            | Yes            | 1             |
| 4   | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes            | Yes            | Yes            | Yes            | Yes            | Yes            | Yes           | Yes           | Yes           | Yes           | Yes            | Yes            | Yes            | Yes            | Yes            | 1             |
| 5   | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes            | Yes            | Yes            | Yes            | Yes            | Yes            | Yes           | Yes           | Yes           | Yes           | Yes            | Yes            | Yes            | Yes            | Yes            | 2             |
| 6   | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes            | Yes            | Yes            | Yes            | Yes            | Yes            | Yes           | Yes           | Yes           | Yes           | Yes            | Yes            | Yes            | Yes            | Yes            | 1             |
| 7   | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes            | Yes            | Yes            | Yes            | Yes            | Yes            | Yes           | Yes           | Yes           | Yes           | Yes            | Yes            | Yes            | Yes            | Yes            | 2             |
| 8   | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes            | Yes            | Yes            | Yes            | Yes            | Yes            | Yes           | Yes           | Yes           | Yes           | Yes            | Yes            | Yes            | Yes            | Yes            | 1             |
| 9   | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes            | Yes            | Yes            | Yes            | Yes            | Yes            | Yes           | Yes           | Yes           | Yes           | Yes            | Yes            | Yes            | Yes            | Yes            | 1             |
| 10  | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes           | Yes            | Yes            | Yes            | Yes            | Yes            | Yes            | Yes           | Yes           | Yes           | Yes           | Yes            | Yes            | Yes            | Yes            | Yes            | 1             |

Abbreviations: SHV = Staphylococcus aureus, VSS = Viridans streptococci.