HEMOLYTIC ACTIVITY OF STAPHYLOCOCCUS HYicus
AND STAPHYLOCOCCUS CHROMOGENES

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Abstract

Skalka B.: Hemolytic activity of Staphylococcus hyicus

Hemolytic activity of 135 S. hyicus and 75 S. chromogenes
strains was examined on sheep, rabbit, human, horse and chicken
blood agars. The agar bases were supplemented with 5 % washed
erthrocytes of the corresponding animal species. The strains
of both staphylococcal species under study produced a wide zone
of complete hemolysis on rabbit blood agar and a narrow one on
media containing horse erythrocytes. The hemolytic effects always
appeared after 48 h incubation at 37°C. Neither species produced
hemolysis on agar containing sheep or chicken erythrocytes, and
no synergistic hemolytic effect was observed on sheep blood agar.
A direct hemolysis was exhibited by 92 (68 %) S. hyicus strains
on human blood agars. The remaining S. hyicus strains and S.
chromogenes did not produce hemolysis of human erythrocytes.
Hemolytic effects of the staphylococci used as controls were
expressed just after 24 h incubation on all kinds of blood agar.
We recommend inclusion of the hemolysis of rabbit erythrocytes
among the characteristic properties of S. hyicus and S.
chromogenes.

Staphylococcus hyicus, Staphylococcus chromogenes, hemolytic
activity, blood agar, sheep, rabbit, human, horse and chicken
erthrocytes.

Staphylococcus hyicus (Baird-Parker 1965), originally named
Micrococcus hyicus (Sompolinsky 1953), was subdivided in two
subspecies, namely subsp. hyicus and subsp. chromogenes (Devriese
et al. 1978; Kloos and Schleifer 1986), but these were later
elevated to species status: S. hyicus and S. chromogenes (Hájek et al.
1986). Lack of hemolysis of sheep red blood cells is one of the most important
properties of both S. hyicus and S. chromogenes (Devriese 1977; Devriese
et al. 1978; Hájek et al. 1986; Kloos and Schleifer 1986; Skalka 1987). It is well established that intact
sheep erythrocytes are relatively insensitive to hemolysins elaborated by
staphylococci of both coagulase-positive and coagulase-negative species (Marks
and Vaughan 1950; Bernheimer et al. 1968; Wieseman 1975). Nevertheless, their subceptibility increases remarkably
if the cell membrane sphingomyelin is disintegrated by the beta-toxin of
S. aureus (Christian and Graydon 1941; Adamczyk and
Blaurock 1963; Skalka et al. 1979a; 1979b; Boyce 1985), or by the toxin of Corynebacterium pseudotuberculosis (Soupček et al. 1967; Skalka et al. 1979b; 1980). Such a phospholipase-conditioned hemolysis, called synergistic hemolysis, is characteristic of staphylococcal delta-lysin (Christie and Graydon 1941; Adamczyk and Blaurock 1963). Hébert and Hancock (1985) and Watts and Owens (1987) observed some synergistic hemolytic phenomenon produced by a limited number of S. hyicus and S. chromogenes strains. On the other hand, the red blood cells of other animal species show greater susceptibility to some bacterial hemolytic substances, so that human (Wiseman and Caird 1968; Wiseman 1975; Kloos and Schleifer 1986), rabbit (Cooper et al. 1964; Bernstein 1965; Wiseman 1975; Fackrell and Wiseman 1976), horse (Guyonnet and Plommet 1970), and poultry erythrocytes (Möllby and Wadström 1971) are recommended for detection of the hemolysins produced by staphylococci.

The present paper is devoted to study the behaviour of S. hyicus and S. chromogenes in media supplemented with red blood cells of different animal species and to examine the possible synergistic hemolysis of S. hyicus and S. chromogenes against sheep erythrocytes.

Materials and Methods

Agar medium
Brain heart infusion agar CM 375 (Oxoid Ltd) was used.

Erythrocytes
Sheep, rabbit, human, horse and pultry (Gallus domesticus) erythrocytes were used. For detection of hemolysis, the agar base described above was supplemented with 5 % (v/v) erythrocytes, washed three times, of the corresponding animal species.

Bacteria
The identification procedure described by Kloos and Schleifer (1986) was used for all staphylococcal strains.

Strain for the synergistic (phospholipase-conditioned) hemolysis
Staphylococcus aureus CCM 6188 producing beta toxin (phospholipase C) was employed for disintegration of sphingomyelin of sheep erythrocytes.

Investigated organisms
The assays were carried out with 135 Staphylococcus hyicus strains, including CCM 2368, 75 Staphylococcus chromogenes strains, including CCM 3387, and 5 strains which were not allocated to any staphylococcal species. The properties of the strains of both species corresponded with those described by Kloos and Schleifer (1986) and Hájek et al. (1986), except that 46 strains of S. hyicus were non-proteolytic and only 38 S. chromogenes strains produced pigment. The non-allocated strains shared characteristics of S. hyicus, namely the strong Tween 80 splitting and the proteolytic activity, but they did not elaborate hyaluronidase.

Control organisms
The following staphylococcal species were used for control purposes: 10 strains of S. aureus, including CCM 2351 producing alpha-toxin, and CCM 2512 producing delta-hemolysin; 10 strains of S. epidermidis, including CCM 2124; 8 strains of S. haemolyticus, including CCM 2737; 3 strains of S. caprae; 5 strains of S. hominis, including CCM 3474; 6 strains of S. simulans, including CCM 2705; 3 strains of S. wernerii, including CCM 2730.
at 37°C, and the result was read after 24 h, 48 h, and 72 h. The direct hemolysis and the hemolytic effect on phospholipase-treated erythrocytes were evaluated on sheep blood agar as described previously (Skała et al. 1979a; 1979b). The other blood agars served merely for demonstration of the direct hemolysis.

**Results**

Three *S. aureus* strains, including CCM 2351, proved to be strongly hemolytic on sheep blood agar, as appeared in the direct effect, but the wide hemolysis surrounding the streaks was reduced in the zone of the beta-hemolysin. Such antagonism of hemolysis is characteristic of those *S. aureus* strains which produce alpha-toxin and only a negligible amount of delta-hemolysin. Seven *S. aureus* strains, including CCM 2512, produced narrower, sometimes indistinct, hemolytic zones as a direct effect, and these zones were always considerably enhanced when erythrocytes were affected by the beta-hemolysin. This synergistic hemolysis is characteristic of staphylococcal delta-hemolysin. The hemolytic activity of 7 *S. epidermidis* strains, 8 *S. haemolyticus* 2 *S. caprae*, 2 *S. hominis*, 2 *S. simulans*, and 1 *S. warneri* strain was the same as of *S. aureus* delta-hemolysin producing strains. These hemolytic effects were apparent just after 24 h incubation (Fig. 1). The remaining strains of coagulase-negative staphylococcal species grew without any direct or synergistic hemolysis (non-hemolytic group - NHG) on sheep blood agar. The strains of *S. hyicus*, *S. chromogenes*, and of the non-allocated staphylococci did not show direct or synergistic hemolysis on sheep blood agar after 24 h, 48 h, and 72 h incubation (Fig. 1). However, after 48 to 72 hours' incubation, a narrow zone of incomplete clearing appeared around inoculation streaks of some strains under study in the zone of activity of the staphylococcal beta-hemolysin. The occurrence of this phenomenon was occasional and its reproducibility was very low.

A wide zone of complete hemolysis appeared around the streaks of three alpha-toxin positive *S. aureus* strains on rabbit blood agar after 24 h incubation, and the diameter of the hemolytic effect increased considerably with further incubation (Fig. 2; Fig. 3). The remaining control strains of *S. aureus* and those of coagulase-negative species, which were hemolytic on sheep blood agar, also hemolyzed rabbit blood agar, producing narrower zones of hemolysis than the
alpha-toxin strains, with only a slight increase in diameter on prolonged incubation (Fig. 2; Fig. 3). The NHG strains did not produce hemolysis on rabbit blood agar. An unusual phenomenon was observed when the strains of S. hyicus, S. chromogenes, and of the group of non-allocated staphylococci grew on rabbit blood agar. No hemolysis appeared around the streaks of the strains under study after 24 h incubation (Fig. 2; Fig. 4). However, a broad zone of complete hemolysis was produced by all strains within a further 24 h (Fig. 3; Fig. 5). This singular delayed hemolysis, appearing after 48 h incubation, was reproducibly shown by all strains under study.

A complete hemolysis of human erythrocytes developed around the growth of S. aureus strains and of coagulase-negative staphylococci, which were hemolytic on sheep and rabbit blood agars. The effect was shown after 24 h incubation. The zone of clearing produced by three alpha-toxin strains was less intense than that caused by the producers of delta-hemolysin and the coagulase-negative strains with hemolytic properties (Fig. 6). The NHG strains did not hemolyze on human blood agar. The hemolytic reaction was seen with 92 (68 %) strains of S. hyicus after 48 h incubation. The hemolysis was not as wide as the zones produced by other hemolytic staphylococci (Fig. 6). The remaining strains of S. hyicus, and all strains of S. chromogenes, did not show hemolysis on human blood agar.

On agar containing horse erythrocytes, a wide zone of complete hemolysis with sharp edges surrounded as the streaks of delta-hemolysin S. aureus strains, as the growth of the hemolytic coagulase-negative staphylococci. A smaller zone with blurred edges surrounded the streaks of three alpha-toxin strains. Both effects were developed after 24 h incubation. S. hyicus, S. chromogenes, and the non-allocated strains produced a very narrow complete hemolysis on this medium after 48 h incubation (Fig. 7). The NHG strains grew without hemolysis on horse blood agar.

All hemolytic strains of staphylococci used as controls produced wide zone of complete hemolysis on agar containing chicken erythrocytes. S. hyicus, S. chromogenes, the non-allocated strains, and the NHG staphylococci grew without hemolysis on this blood agar (Fig. 8).
**Discussion**

The detection of hemolytic activity, especially on sheep blood agar, is a significant component of detailed diagnosis of many bacterial species, or of some strains within a species. The production of hemolysin(s) provides an important marker in the identification of staphylococci. In view of this fact, it is therefore important to specify which of the known hemolysins is produced (Marks and Vaughan 1950; Adamczyk and Blaurock 1963; Bernheimer et al. 1968; Wiseman 1975; Skalka et al. 1979a; 1979b; 1980; Boyce 1985; Hébert and Hancock 1985; Kloos and Schleifer 1986; Watts and Owens 1987). Nevertheless, hemolytic inactivity constitutes a feature of diagnostic value, for example for *S. hyicus* and *S. chromogenes* (Devriese et al. 1978; Hájek et al. 1986; Kloos and Schleifer 1986). Some strains of *S. aureus*, however, especially those of the ecovar B, do not produce hemolysin either, and lack of hemolysis of some *S. intermedius* strains has also been described (Skalka et al. 1979a; 1980). In addition, hemolytic strains in many coagulase-negative species are known (Christie and Graydon 1941; Hébert and Hancock 1985; Kloos and Schleifer 1986; Watts and Owens 1987). Hemolysis of those strains on sheep blood agar is usually weak, but becomes stronger if red blood cells of other animal species (Marks and Vaughan 1950) or the synergistic (phospholipase-conditioned) hemolysis (Adamczyk and Blaurock 1963) are employed.

The hemolytic effects of coagulase-positive and coagulase-negative staphylococci used for control in the present study agreed with the descriptions of other authors (Marks and Vaughan 1950; Jackson 1962; Adamczyk and Blaurock 1963; Cooper et al. 1964; Bernheimer 1965; Wiseman 1975; Wiseman and Caird 1976; Boyce 1985; Hébert and Hancock 1985; Watts and Owens 1987) and with our previous experience as well (Skalka et al. 1979a; 1979b; 1980).

The inability of *S. hyicus* and *S. chromogenes* to produce the direct hemolysis of the intact sheep red blood cells (Sompolinsky 1953; Baird-Parker 1965; Devriese 1977; Devriese et al. 1978; Hájek et al. 1986; Kloos and Schleifer 1986)
Fig. 1. Agar with sheep erythrocytes (S). Vertical streak of S. aureus producing beta-hemolysin (B). Horizontal streaks: S. aureus producing alpha-toxin (A); S. hyicus (H); S. aureus producing delta-hemolysin (D); S. chromogenes (C). Effects after 48 h incubation.

Fig. 2. Agar with rabbit erythrocytes (R). Streaks of growth: S. aureus producing alpha-toxin (A); S. hyicus (H); S. aureus producing delta-hemolysin (D). Hemolysis after 24 h incubation.
Fig. 3. Agar with rabbit erythrocytes (R). Streaks of growth: S. aureus producing alpha-toxin (A); S. hyicus (H); S. aureus producing delta-hemolysin (D). Hemolysis after 48 h incubation.

Fig. 4. Agar with rabbit erythrocytes (R). Streaks of growth: two different strains of S. hyicus (H 1; H 2); S. chromogenes (C). Growth after 24 h incubation.
Fig. 5. Agar with rabbit erythrocytes (R). Streaks of growth: two different strains of S. hyicus (H 1; H 2); S. chromogenes (C). Hemolysis after 48 h incubation.

Fig. 6. Agar with human erythrocytes (M). Streaks of growth: S. aureus producing alpha-toxin (A); S. hyicus (H); S. aureus producing delta-hemolysin (D). Hemolysis after 48 h incubation.
Fig. 7. Agar with horse erythrocytes (E). Streaks of growth: S. aureus producing alpha-toxin (A); S. hyicus (H); S. aureus producing delta-hemolysin (D). Hemolysis after 48 h incubation.

Fig. 8. Agar with poultry erythrocytes (P). Streaks of growth: S. aureus producing alpha-toxin (A); S. hyicus (H); S. aureus producing delta-hemolysin (D). Hemolytic effects after 48 h incubation.
wasconfirmed by us in the present paper. In addition, we did not observe a marked hemolytic effect when strains of both species were assayed on sheep erythrocytes treated by staphyloccocal beta-toxin. A positive synergistic hemolysis of S. hyicus and S. chromogenes was described (Hébert and Hancock 1985; Watts and Owens 1987), and the authors took for granted the production of delta-hemolysin (Hébert and Hancock 1985) or of cytolysin, which acts synergistically with S. aureus beta-toxin (Watts and Owens 1987), by some strains of both species. A narrow zone of incomplete clearing around streaks of some strains under study, observed by us in synergistic hemolytic assays, resembles the description of Devriesse (1977), who described as hemodigestion the direct effect of some S. hyicus strains on sheep blood agar, explaining it as a consequence of strong proteolytic activity. A close relation of proteolytic activity and the clearing phenomenon observed by us could not be shown, because even some non-proteolytic S. hyicus strains showed this phenomenon. On the contrary, a great number of proteolytic strains did not produce it.

The use of rabbit erythrocytes in the detection of hemolytic properties of S. hyicus and S. chromogenes has not been previously described. These red blood cells were hemolyzed by all strains of the both species, and the hemolysis appeared generally after 48 h of incubation. Such delayed hemolysis distinguishes S. hyicus and S. chromogenes from the alpha- and delta-hemolysin producing S. aureus strains and from the coagulase-negative staphylococci with hemolytic properties. Strains of these groups always produce hemolysis on rabbit blood agar just after 24 h incubation.

The effects shown by the control organisms on media containing sheep, rabbit, human, horse, and poultry erythrocytes corresponded with the characteristic activity of staphyloccocal hemolysins alpha and delta (Cooper et al. 1962; Jackson 1962; Bernheimer 1965; Bernheimer et al. 1968; Wiseman and Caird 1968; Wiseman 1975). The hemolysis of S. hyicus and S. chromogenes resembled the activity of staphyloccocal gamma-hemolysin (Jackson 1962; Guyonnet and Plommet 1970; Möllby and Wadsström 1971; Fackrel and Wiseman 1976). The production of gamma-hemolysin by S. hyicus and S. chromogenes was described (Goodfellow et al. 1987). But gamma-hemolysin produces a complete hemolysis
of sheep erythrocytes and its activity is apparently inhibited by agar (Jacks on 1962). The nature of hemolytic activity of S. hyicus and S. chromogenes, described in the present study, thus remains to be explained, although relation to the staphylococcal gamma-hemolysin is perhaps possible.

The strains previously non-allocated to S. hyicus because of their failure to produce hyaluronidase displayed hemolytic patterns like those of S. hyicus. We suggest that these strains are S. hyicus and presume that a limited number of hyaluronidase-negative strains exists in this species.

The hemolytic activity of S. hyicus and S. chromogenes on rabbit blood agar is a useful contribution to identification of both staphylococcal species, and we recommend inclusion of the hemolysis of rabbit erythrocytes among the characteristic properties of S. hyicus and S. chromogenes. We propose that the hemolyses described in this paper are important markers of S. hyicus and S. chromogenes.

Hemolytická aktivita Staphylococcus hyicus a Staphylococcus chromogenes

Hemolytická aktivita 135 kmenů S. hyicus a 75 kmenů S. chromogenes se sledovala na krevních agarech s ovčími, králičími, lidskými, koňskými a aviárními (kur domácí) erytrocyty. Agarové medium obsahovalo vždy 5% propraných erytrocytů příslušného druhu. Všechny kmeny obou sledovaných stafylokokových druhů hemolyzovaly intenzivně králičí erytrocyty a jen velmi slabě koňské. Hemolýzy byly zřetelné vždy až po 48 h inkubaci při 37°C. Na krevních agarech s ovčími a aviárními erytrocyty vyrůstaly kmeny obou sledovaných stafylokokových druhů bez náznamu hemolýzy, která se na mediích s ovčími krvinkami nepozorovala ani v testu synergismu se stafylokovým beta hemolyzinem. Na krevních agarech s lidskými erytrocyty hemolyzovalo 92 (68%) kmenů S. hyicus, ostatní kmeny tohoto druhu byly nehemolytické, stejně jako všechny kmeny S. chromogenes. Kontrolně použité kmeny S. aureus, produkující hemolýzy alfa, nebo delta, hemolyzovaly na všech krevních agarech již po 24 h inkubaci, stejně jako hemolytické kmeny koaguláza negativních stafylokoků. Vyslovuje se doporučení zahrnout prolongovanou hemolytickou aktivitu na krevních agarech s králičími erytrocyty do komplexu charakteristických vlastností S. hyicus a S. chromogenes.
Гемолитическая активность Staphylococcus hyicus и Staphylococcus chromogenes

Гемолитическую активность 135 штаммов S. hyicus и 75 штаммов S. chromogenes исследовали на агарах крови овец, кроликов, человека, лошадей и птиц. Агар содержал всегда 5% промытых эритроцитов соответствующего вида. Все штаммы обоих исследуемых стафилококковых видов интенсивно гемолизировали агари кроликов, весьма слабо — агари лошадей. Гемолиз отчетливо выделялся всегда через 2 суток после инкубации. На овечьих и птичьих агарах крови вырастили штаммы обоих видов без признаков гемолиза, не наблюдаемого в случае агара овец даже в тесте синергизма со стафилококковым бета-токсином. На агарах с человеческими эритроцитами был установлен гемолиз 68% штаммов S. hyicus, остальные штаммы данного вида отличались агемолитичностью как и все остальные штаммы S. chromogenes. Вырабатывющие гемолизы альфа или дельта контрольные штаммы S. aureus гемолизировали на всех аграх уже через 1 сутки после инкубации как и штаммы коагулаzo негативных стафилококков. Рекомендуется включить продленную гемолитическую активность на агарах крови кроликов в комплекс характерных свойств S. hyicus и S. chromogenes.

References
