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Haemolytic activities of *Trichophyton* species

P. SCHAUFUSS & U. STELLER
Serumwerk Memsen, Memsen, Hoyerhagen, Germany

Dermatophytes are keratinophilic fungi able to invade the stratum corneum of the skin and other keratinized structures. The pathogenic interactions between host and fungus are poorly understood. Some enzymes, especially keratinases, have previously been taken into consideration as virulence factors. Haemolysins have not been evaluated in this regard, though they are known to play an important role in the host–parasite interaction in bacterial infections. We investigate the haemolytic activity of four *Trichophyton* species: *T. rubrum*, *T. mentagrophytes* complex, *T.equinum* and *T. verrucosum.* The strains were tested on Columbia agar with 5% blood from horses, cattle and sheep. They show different haemolytic activities. *T. rubrum* and *T. equinum* produce a zone of complete haemolysis followed by a small zone of incomplete haemolysis around the colony. *T. mentagrophytes* and *T. verrucosum* produce a zone of complete haemolysis. Haemolytic activity is pronounced in dermatophytes and may play an important role as a virulence factor.

Keywords dermatophytes, haemolysis, *Trichophyton* species

Introduction

Among the aetiological agents of dermatophytosis, some *Trichophyton* species are commonly described in connection with human and animal diseases. Dermatophytes are keratinophilic fungi that are able to invade the stratum corneum of the skin as well as other keratinized structures [1]. Usually, dermatophytes are unable to penetrate deeper tissues [2,3]. *Trichophyton rubrum*, one of the most important species, is commonly aetiological in humans; transmission occurs from person to person [4]. *Trichophyton mentagrophytes sensu lato*. (i.e. as defined in the broad, morphotaxonomic sense of the name) is one of the most common dermatophytes infecting humans and animals. Human infections caused by granular-type isolates often follow contact with infected animals [5]. *Trichophyton equinum*, a zoophilic species, is primarily isolated from horses or their human handlers [6] while *Trichophyton verrucosum*, also zoophilic, causes ringworm in cattle, and poses an infection risk for people handling these animals [7].

Host–pathogen interactions in dermatophytosis are poorly understood. Some exocellular enzymes that diffuse through the cornified skin layer during infections have been taken into consideration as important virulence factors [8]. These enzymes include keratinase [9–11], elastase [12], collagenase [13] and lipase [14].

Haemolysins play an important role in the infectious process in bacterial infections [15]. The haemolysin of *Listeria monocytogenes* was the first bacterial gene product found to be necessary for the survival of this bacterium within eukaryotic host cells [16]. Haemolysins have been described as having cytotoxic effects on the membranes of erythrocytes [17] and phagocytic cells [18], as well as pore-forming and lysis effects on other eukaryotic cells and cellular structures [19,20]. Various haemolysins differ in their biochemical and cytotoxic properties [15].

The object of this study was to investigate the haemolytic activities of the *Trichophyton* species listed above.

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Correspondence: Dr Peter Schaufuß, Serumwerk Memsen, Memsen 13, D-27318 Hoyerhagen, Germany. Tel.: +49 4251 930921; Fax: + 49 4251 930949; E-mail: dr.schaufuss@t-online.de

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Materials and methods

Strains

The strains used in this study were obtained from the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany, and from the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands. The following strains were used: *T. rubrum* DSM 4167, CBS 202.88 and CBS 389.58. T. *mentagrophytes* DSM 4870, CBS 263.79 and CBS 388.58; T. *equinum* DSM 12284, CBS 270.66 and CBS 109033; and *T. verrucosum*, DSM 7380, CBS 365.53 and CBS 562.50. The strains were grown on Sabouraud glucose agar (Oxoid CM41; Unipath, Basingstoke, UK) and controlled for purity and identity [21].

Haemolytic activity assay

Haemolytic activity was controlled by seeding of the strains onto Columbia 5% blood agar (C-BAP; Oxoid CM331). Blood was taken from horses, bovines and sheep. *T. rubrum*, *T. mentagrophytes* and *T. equinum* were incubated under aerobic conditions at 27°C for 7–14 days, *T. verrucosum* was incubated at 27°C for 19–30 days. In order to increase the haemolytic activity, the cultures were transferred to an incubator at 36°C for 1–5 days. Haemolytic activity was monitored every day.

Test for saprotrophic or bacterial contamination

After monitoring of haemolytic activity, subcultures were taken from the C-BAP and seeded on C-BAP, Gassner agar (Merck 1282; Darmstadt, Germany) and Sabouraud glucose agar. The C-BAP and Gassner agars were incubated at 36°C for 4 days. The Sabouraud glucose agar was incubated at 27°C for 14 days and examined every day. No bacterial contamination could be detected.

Results

Trichophyton rubrum showed cottony colonial morphology after 3–7 days of incubation at 27°C on C-BAP amended with bovine blood. The colony was typically surrounded by a zone of wine-red colour (Fig. 1a). The reverse side of young colonies was dark brown, and was also surrounded by the wine-red zone. After about 6–10 days further incubation, a zone of incomplete haemolysis was detected on the reverse (Fig. 1b). The zone of wine-red colour decreased in proportion to the increase in the haemolysed zone. To increase the haemolytic activity, the strains were incubated at 36°C for an additional 4–5 days. Cultures produced a strong zone of complete haemolysis surrounded by a small zone of incomplete haemolysis (Fig. 1c). Nearly the same results were obtained on C-BAP made up with sheep or horse blood.

Trichophyton mentagrophytes cottony, velvety and granular-type colonies grew rapidly on all types of C-BAP. After 2–6 days incubation at 27°C, young colonies appeared (Fig. 2a). Their reverse was dark-red to brown, and older colonies become dark brown. In contrast to what was seen with *T. rubrum*, a zone of complete haemolytic activity could be first recognized on the reverse side after about 7–11 days at 27°C (Fig. 2b). After 1–2 days incubation at 36°C, the haemolysis was much more intense than that seen with *T. rubrum* (Fig. 2c). We could not find any differences in haemolytic activity among the colony forms, nor could an influence of the different blood supplements be observed.

After 6–10 days incubation at 27°C, velvety growth of *T. equinum* could be detected. Colonies produced a yellow to brown pigment at the centre (Fig. 3a) that differed in intensity among different strains. Colony reverses showed dark brown centres, surrounded by a yellow zone (Fig. 3b). As with *T. rubrum*, young colonies produced a small zone of incomplete haemolysis. After a further incubation period of 3–8 days at 36°C, *T. equinum* colonies produced a small zone of complete haemolysis with all types of blood used (Fig. 3c).

Trichophyton verrucosum grew very slowly, but sufficient growth to permit analysis appeared on C-BAP after 15–20 days at 27°C (Fig. 4a). Colonies were yellow to brown on the reverse, and were surrounded by a small zone of complete haemolysis (Fig. 4b). As with the other species tested, incubation at 36°C was found to increase the amount of haemolytic activity directed against all three types of blood (Fig. 4c). No differences in haemolytic activity among the different strains within each species used could be detected.

Discussion

C-BAP is a basal medium to which blood is added for isolating and cultivating fastidious microorganisms, mainly bacteria. The medium combines two types of peptones, a casein hydrolysate and a meat infusion, to promote rapid, luxuriant growth and sharply defined haemolytic reactions, as well as typical colonial morphology and improved pigment production in bacteria [22]. We used this medium, adding blood from horses, bovines or sheep, and obtained parallel results with dermatophytes. For the demonstration of haemolytic

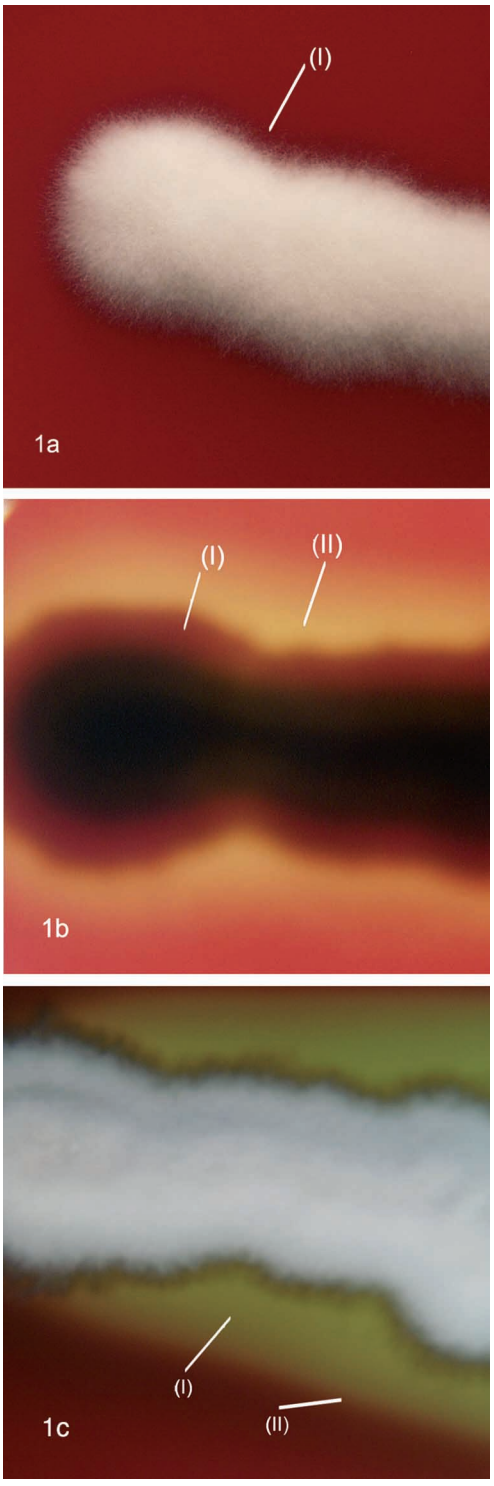

Fig. 1 *Trichophyton rubrum* DSM 4167 on Columbia bovine blood agar. (a) Cottony form, 5 days/27°C. The colony is surrounded by a small zone of wine-red colour (I). (b) Reverse side, 6 days/27°C. The middle of the colony is dark brown, surrounded by a zone of wine-red colour (I), followed by a zone of incomplete haemolysis (II). (c) Cottony form, 7 days/27°C and 4 days/36°C. The zone of complete haemolysis (I) is surrounded by a zone of incomplete haemolysis (II).

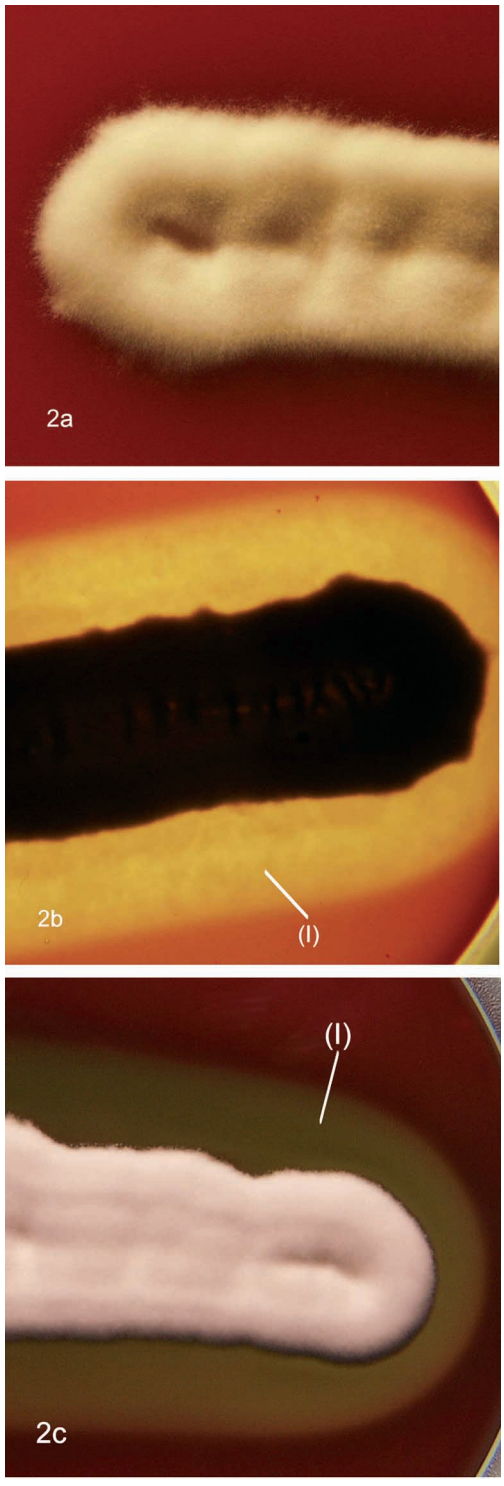

Fig. 2 *Trichophyton mentagrophytes* DSM 4870 on Columbia sheep blood agar. (a) Cottony form, 6 days/27°C. (b) Reverse side, 11 days/27°C and 1 day/36°C. The colony is surrounded by a zone of complete haemolysis (I). (c) Cottony form, 11 days/27°C and 2 days/36°C. The colony is surrounded by a strong zone of complete haemolysis (I).

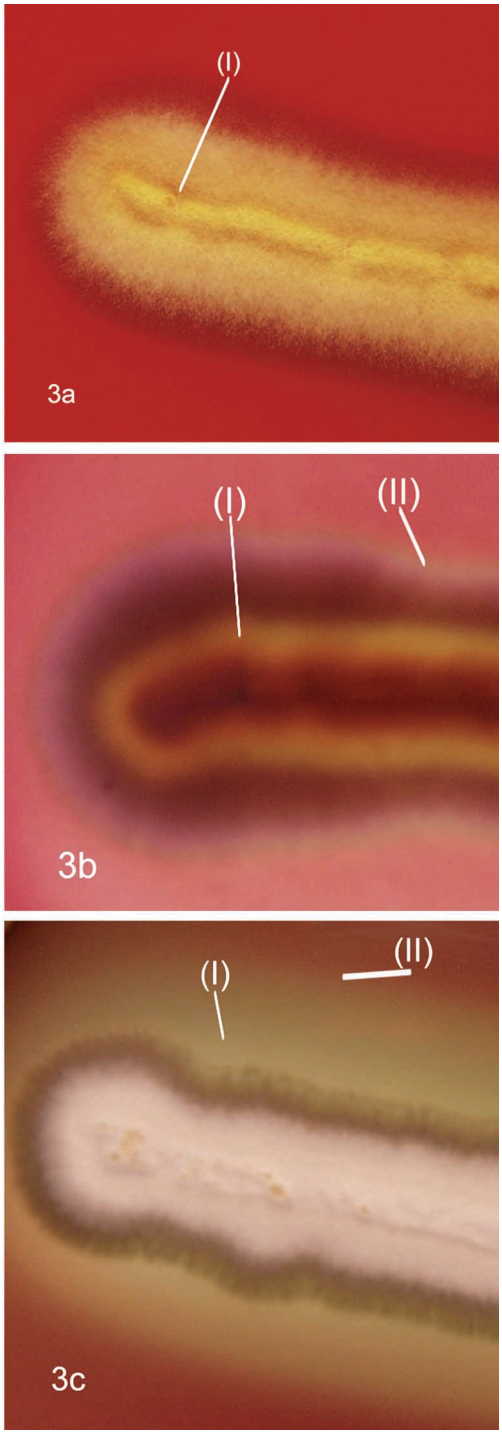

Fig. 3 *Trichophyton equinum* DSM 12284 on Columbia sheep blood agar. (a) Velvety form, 6 days/27°C, producing a yellow to brown pigment in the middle of the colony (I). (b) Reverse side, 7 days/27°C. The centre of the colony is dark brown surrounded by a zone of yellow pigmentation (I), followed by a zone of incomplete haemolysis (II). (c) Velvety form, 6 days/27°C and 8 days/36°C. The colony is surrounded by a zone of complete haemolysis (I) followed by a zone of incomplete haemolysis (II).

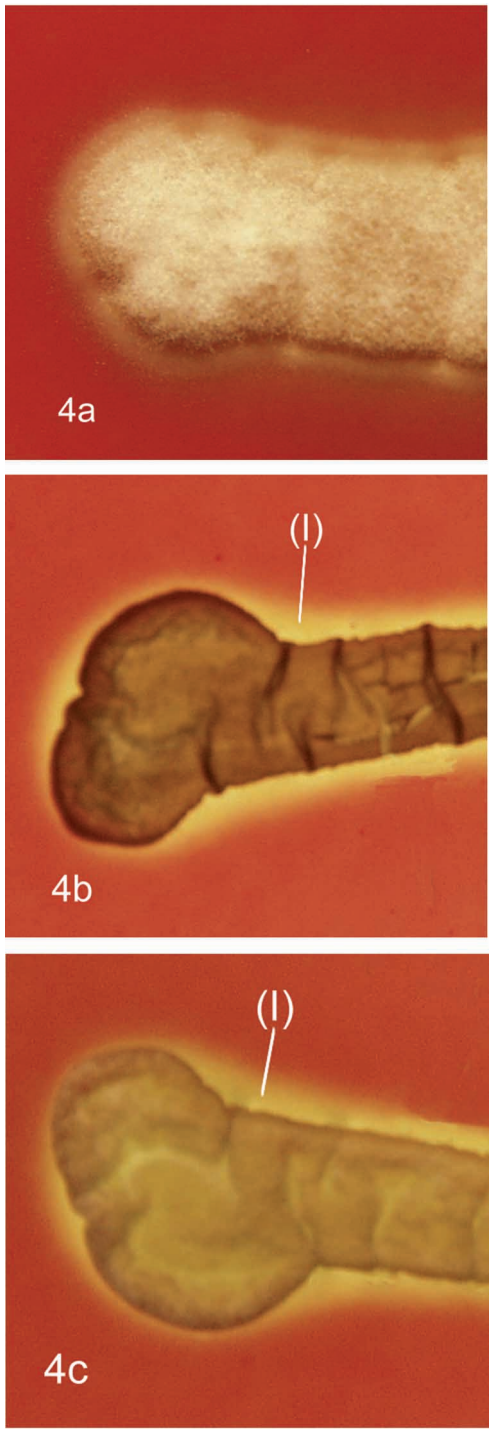

Fig. 4 *Trichophyton verrucosum* DSM 7380 on Columbia bovine blood agar. (a) Colony of *T. verrucosum*, 19 days/27°C. (b) Reverse side, 19 days/27°C and 1 day/36°C. The colony is yellow to brown, surrounded by a small zone of complete haemolysis (I). (c) Colony of *T. verrucosum*, 19 days/27°C and 1 day/36°C, surrounded by a zone of complete haemolysis (I).

activities of the *Trichophyton* species tested, the size of the colonies was found to be very important. In contrast to bacteria, fungi have a very slow growth rate. *T. rubrum*, *T. mentagrophytes* and *T. equinum* needed 7 days and *T. verrucosum* needed 15 days to form colonies sufficiently large (> 10 mm diameter) to yield definite results. An additional 1–6 days at 36°C were found to be necessary for the production of readily visible zones of haemolysis. During this time, there is always the risk of autolytic processes occurring within the C-BAP. For this reason, the demonstration of haemolysis in a single colony is nearly impossible. Large multi-colonial masses of fungi produce a higher amount of haemolysin than individual colonies do, and they also require less time to produce discrete, visually detectable levels.

In various bacteria, haemolysins are one of the most important virulence factors mediating the severity of infections in vertebrates [16]. The loss of haemolytic activity often results in avirulence [23]. Different kinds of haemolysins have been described. *Listeria monocytogenes* produces a cholesterol-dependent, pore-forming toxin, CDTX, listeriolysin [24], while *Streptococcus pyogenes* produces streptolysin O, a compound with a similar mechanism of action [25]. These cytolysins, along with the α-haemolysin produced by *Staphylococcus aureus*, belong to a group of channel-forming proteins [20] that interact with phagocytic cells and phagosome membranes [18]. Another group of cytolysins belonging to a heterogeneous group of enzymes called 'phospholipases' share the ability to hydrolyse ester linkages in glycerophospholipids of host cell membranes, giving them potential influence on the virulence of fungi and bacteria [26]. Lipase and phospholipase activities are also shown by different *Trichophyton* species [14,27]. On the other hand, phospholipase activity alone does not explain haemolytic activities. The question of whether the phospholipases are an integral part of the *Trichophyton* lytic apparatus or a latent enzyme of the erythrocyte that is activated by extracellular secretions of the *Trichophyton* species is not clear [28]. It should be noted that the addition of pure phospholipases to erythrocyte suspensions does not result in haemolysis; rather, the activity of a special co-factor is necessary [29].

Haemolytic activities levels in dermatophytes have been shown to correlate with severity and chronicity of clinical infections, but in these studies the type of erythrocytes used was not specified [30]. It is well known that erythrocytes of different mammalian species vary in their cell membrane compositions [31] and have specific phospholipid constituents [29]. No study to date has directly addressed this matter in connection with dermatophyte enzymes, and thus the exact mode of action of *Trichophyton* haemolysins remains unknown.

Our findings showed a bizonal lytic effect in *T. rubrum* and *T. equinum* strains after cultivation on C-BAP with sheep, bovine and horse blood. This effect consisted of a zone of complete haemolysis surrounded by a zone of incomplete haemolysis, indicating secretion of two different cytolytic factors. On the other hand, the colonies of *T. mentagrophytes* and *T. verrucosum* were surrounded by just a single zone of complete haemolysis. The present publication gives only an overview about the haemolytic activities of *Trichophyton* species. A separate manuscript will describe the nature of haemolytic activity in *Microsporum canis*. More investigations concerning related biochemical and cytotoxic properties will be necessary.

Generally, as mentioned above, infections with *Trichophyton* species are cutaneous, because of the inability of these fungi to penetrate deep tissues of immunocompetent patients [2,3]. On the other hand, immunological reactions of the host to metabolic products of the fungi can result in mild to intense inflammation. The immunological reaction is humoral and cell-mediated, it consists of the immigration of lymphocytes, macrophages, neutrophils and mast cells into the skin [32]. Bacterial haemolysins are well known to be toxic to these cells. Haemolysins produced by *Trichophyton* species may similarly play an important role in the balance between the host's cellular immunity and the ability of the fungus to diminish the immune response.

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