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Isolation and characterization of a haemolysin from
Trichophyton mentagrophytes

P. Schaufuss¹, F. Müller² and P. Valentin-Weigand²

¹Serumwerk Memsen WDT, Memsen 13, 27318 Hoyerhagen, Germany

²Institut für Mikrobiologie, Zentrum für Infektionsmedizin,
Tierärztliche Hochschule Hannover, Bischofsholer Damm 15,
30173 Hannover, Germany

Corresponding author:

Dr. Peter Schaufuss

Serumwerk Memsen WDT

Memsen 13

27318 Hoyerhagen, Germany

Phone: + 49 4251 930920

Fax: + 49 4251 930949

E-mail: schaufussdr@aol.com

Abstract

Haemolytic activities of *Trichophyton (T.) mentagrophytes* were detected and characterized by qualitative and quantitative assays. On Columbia agar supplemented with blood from horses, cattle or sheep, *T. mentagrophytes* expressed a strong zone of complete haemolysis. No haemolytic activities could be detected in the closely related *T. verrucosum* var. *ochraceum*. The same results were obtained after cultivation of the fungi on sterile cellulose acetate filters placed on the surface on Columbia blood agar. After removal of the filter, complete haemolysis was detected below the colony of *T. mentagrophytes*. A soluble haemolysin from culture supernatant of this strain was isolated and partially purified. Specific haemolytic activity per mg protein was enriched 2.6-fold in filtrate F₁, a fraction obtained as filtrate after filtration through 3 kDa cut-off membranes. The partially purified haemolysin was neither affected by pepsinase K treatment, nor by high and low temperatures, suggesting that it represents a small peptide haemolysin. Accordingly, in a commercial enzymatic activity test only the crude culture filtrate, but none of the subsequent purification fractions showed reactivity. Evaluation of the specificity of the haemolysin using erythrocytes from different mammalian species revealed that sensitivity was highest to those of equines, followed by erythrocytes from sheep, cattle, swine, dogs and humans. None of the erythrocytes was lysed by filtrate F₁ from *T. verrucosum* var. *ochraceum*. Furthermore, different eukaryotic cell lines from different species were tested in their sensitivity to cytolytic activities of the haemolysin, but no membrane damage could be detected.

Keywords Dermatophytes, Haemolysis, *Trichophyton mentagrophytes*

1 Introduction

2
3 Haemolysins produced by different microorganisms represent a group of membrane active toxins
4 (Bernheimer and Rudy, 1986). Many bacterial haemolysins are considered important virulence
5 factors mediating the severity of infections in vertebrates (Vázquez-Boland et al., 2001). Various
6 haemolysins differ in their biochemical and cytotoxic properties (Bernheimer, 1968). For example,
7 staphylococcal α -toxin is produced as a single-chained, nonglycosylated polypeptide by most
8 strains of *Staphylococcus aureus*. The toxin spontaneously binds to lipid monolayers and bilayers,
9 forming hexamers and finally functional transmembrane pores serving as nonphysiologic calcium
10 channels (Bhakdi et al., 1988). The binding of α -toxin to membranes does not require the
11 presence of receptor molecules, in contrast to another group of haemolysins called sulfhydryl-
12 activated cytolsins which specific bind to cholesterol. Streptolysin-O produced by *Streptococcus*
13 *pyogenes* is the prototype of this group of toxins (Bhakdi et al., 1985). Membrane damage results
14 from cholesterol rearrangement after binding to circular structures on membranes leading to
15 disruption of the bilayer (Bhakdi et al., 1984). Haemolytic reactions can also be triggered by a
16 heterogeneous group of enzymes, phospholipases. The members of this group share the ability to
17 hydrolyse ester linkages in glycerolphospholipids of host cell membranes (Ghannoum, 2000). A
18 different group of very small peptide haemolysins, represented by the δ -toxin of *Staphylococcus*
19 *aureus*, produce cell lyses by forming tetrameric and higher aggregates in aqueous solutions
20 (Mellor et al., 1988). Alamethicin, a peptide antibiotic produced by the fungus *Trichoderma viride*
21 (Fringeli and Fringeli, 1979), is another member of this group.

22 In a previous study, we have reported that dermatophytes can trigger haemolytic (Schaufuss and
23 Steller, 2003) as well as co-haemolytic (Schaufuss et al., 2005) reactions, although the underlying
24 mechanisms are yet unknown. The object of this study was to isolate and characterize a
25 haemolysin from *Trichophyton mentagrophytes*.

Materials and Methods

Strains

Trichophyton mentagrophytes strain cbs 388.58 (Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands), isolated from a dog, and *T. verrucosum* var. *ochraceum* strain SM117 (Serumwerk Memsen, Hoyerhagen, Germany), isolated from a calve, were used in this study. The latter was used as negative control in the haemolytic activity assays. The strains were grown on Sabouraud agar containing 4% glucose (Oxoid CM 41; Unipath, Basingstoke, U.K.) for 15 days at 27°C, and controlled for purity and identity as described previously (Schaufuss and Steller, 2003).

Qualitative haemolytic activity assay

A qualitative haemolytic activity assay was performed on Columbia agar, supplemented with 5 % equine (E-BAP), ovine (O-BAP) or bovine (B-BAP) erythrocytes (Oxoid CM 331), which had been washed twice and were then resuspended in saline at pH 7.2 in a twofold concentration. The agar had been poured in 10 ml volumes into plastic plates (15x100 mm). A needle was used for punctiform inoculation of the dermatophyte cultures to be tested as described previously (Schaufuss et al., 2005). Haemolysis was monitored after 11 days at 27 °C.

Quantitative haemolytic activity assay

Sheep erythrocytes were isolated and purified from 50 ml freshly drawn blood by centrifugation at 1200 x g for 10 min at 4 °C and subsequent washings with saline until the supernatant was clear, and were then resuspended in saline to a final concentration of 3 % (w/v). Titrations were run in V-form 96-well microtiter plates (Sarstedt, Newton, USA), starting with 225 µl of the samples to be tested in 25 µl dilution steps to a final volume of 50 µl. Each dilution was filled up to a volume of 250 µl with saline, and subsequently 50 µl of a 3% suspension of washed erythrocytes were added. The plates were incubated at 37 °C for 2 h. Non-lysed erythrocytes were removed by centrifugation at 1000 x g for 5 min. The supernatant was transferred to a flat-bottom, 96-well microtiter plate for spectrophotometric measurements at $A_{550\text{ nm}}$. In controls, samples to be tested

were replaced by saline (negative control) or ddH₂O (positive control). Each experiment was run in triplicate. Results were expressed as % haemolysis (haemolytic activity) as compared to haemolysis by ddH₂O.

Filter test

Both strains were also tested for haemolytic activity by inoculation on sterile cellulose acetate filters (0.2µ, Sartorius AG, Göttingen, Germany), placed directly on the surface of Columbia agar plates, which were supplemented with ovine, bovine or equine erythrocytes as described above. After incubation at 27°C for 7 to 9 days, the filters and the cultures were removed and the blood agar controlled for haemolytic activities.

Partial purification of the haemolysin from culture filtrate

Sterile culture filtrates were obtained by incubation of the dermatophytes in 500 ml Sabouraud 2 % Glucose bouillon (Oxoid CM 147) at 27 °C for 15 days and subsequently sedimented by centrifugation at 4000 x g (Heraeus Multifuge 4 UR, rotor-type LH-4000; Heraeus Instruments, Hanau, Germany). Culture supernatants were filtered for sterilization through a 0.2 µm membrane filter (Sartobran 300 filter, 0.2µm; Sartorius, Göttingen, Germany). A total of 100 ml sterile culture filtrate was concentrated to 10 ml (> 10 kDa, C₁) using an ultrafiltration system (Vivaflow cross-flow system, VF05CO, exclusion limit 10 kDa; Viva-science, Hannover, Germany). The obtained permeate (< 10 kDa, P₁) was concentrated again (>3 kDa, C₂) using another filtration system (Amicon Stirred Cells System, Model 402, YM-Membrane, exclusion limit 3 kDa; Millipore, Bedford, USA). The resulting filtrate (<3 kDa, F₁) was used for further studies.

Protein contents of the culture filtrates and concentrates were determined using a Bio-Rad D_c Protein test system (Bio-Rad D_c Protein Assay, Bio-Rad, München, Germany) in a flat-bottom, 96-well microtiter plate with bovine serum albumin as standard according to Lowry et al., (1951).

SDS-PAGE

Analytical SDS-PAGE was performed by the method of Laemmli (1970), with 4% (v/v) stacking, and 15% (v/v) resolution acrylamide slab gels (Rotiphorese Gel 30, Roth, Karlsruhe, Germany). Molecular weight standards (SeeBlue Pre-Stained Standard, Invitrogen, Carlsbad, USA) were included in each run. The protein bands were stained with silver nitrate (Roth, Karlsruhe) according to Blum et al., (1987).

Determination of enzymatic activities

Culture filtrates, permeates P_1 and filtrate F_1 were also examined semi-quantitatively for the presence of different enzymes using the API-ZYM system (API ZYM, Biomérieux, Hazelwood, USA) as described by Brasch and Zaldua, (1994).

Specificity of haemolytic activity

Specificity of haemolytic activity (using erythrocytes from various species, including sheep, cattle, swine, horses, dogs, and humans), were determined using filtrate F_1 in the quantitative haemolytic activity assay described above. Erythrocytes were isolated and purified from freshly drawn blood from respective animal species, which were kindly provided by our Clinical departments. After washing, the erythrocyte suspensions were adjusted to a concentration of 3 % (w/v) as described above.

In addition, the cytolytic activity of the filtrate F_1 was determined using the lactate dehydrogenase (LDH) release assay (Cytotox 96[®] Assay; Promega, Heidelberg, Germany) according to the manufacturer's instructions. For this, different cell lines, including HEP-2, J774, MDBK, MDCK, and ST cells, were tested after incubation with 1:2, 1:3, 1:6 dilutions of the filtrate made in cell culture media with a final volume of 150 μ l for 3 hours. Cells were cultivated in DMEM (MDCK in EMEM, MDBK in EDulb) (Gibco-Invitrogen, Heidelberg, Germany) supplemented with 10 % fetal calf serum (FCS) (MDBK with 5 % FCS) and 5mM glutamine. Cells were seeded on 24-well plates as described previously (Valentin-Weigand et al., 1996; Benga et al., 2004).

1 **Stability of the haemolysin**

2 The sensitivity of haemolytic activity to proteinase K was tested using proteinase K-acrylic beads
 3 (Sigma, Steinheim, Germany). Prior to use, the beads were briefly hydrated in ddH₂O, adjusted to
 4 a concentration of 0.1 mg/ml, washed three times in saline. The supernatant was discarded, 1 ml
 5 and 300 µl filtrate F₁ were added to 0.1 mg swelled beads and incubated over night at 37°C under
 6 permanent rotation. A volume of 200 µl was then tested qualitatively for haemolytic activity as
 7 described above.

8 The sensitivity to temperature was examined by heating the culture filtrate at 100 °C for 10 min,
 9 and subsequent testing the haemolytic activity. Furthermore, haemolytic activity was tested after
 10 freezing the filtrate at –20°C for 24 h, and after storage at 4°C for 6 month.

11 **Results**

12 *T. mentagrophytes* expressed a zone of complete haemolysis on Columbia agar supplemented
 13 with blood from horses, cattle, or sheep at 27°C after 7 – 10 days of incubation (Fig. 1, A). No
 14 haemolytic activities could be detected around the colonies of *T. verrucosum* var. *ochraceum* (Fig.
 15 1, B). Both fungi strains were also cultivated at 27°C for 7 to 9 days on sterile cellulose acetate
 16 filters on the surface of Columbia agar supplemented with blood from the same animal species.
 17 After removal of the filter membranes, a clear zone of complete haemolysis could be detected only
 18 below the colony of *T. mentagrophytes* (Fig. 1, C and D).

19 A soluble haemolysin was isolated and partially purified from the culture supernatant of *T.*
 20 *mentagrophytes* using a combination of different filtration steps. Haemolytic activity of each step
 21 was controlled in the quantitative haemolysin test. Specific haemolytic activity per mg protein was
 22 enriched 2.6-fold (Tab. 1). Separation of the different purification steps by SDS-PAGE and
 23 subsequent silver stained revealed numerous protein bands in the crude culture filtrate and in the
 24 concentrate C₁ (> 10 kDa) (Fig. 2, A). No bands were detected after separation of the permeate P₁
 25 (< 10 kDa) and the filtrate F₁ (< 3 kDa, data not shown). Similar results were obtained using a
 26 27

1 culture filtrate of *T. verrucosum* var. *ochraceum* (Fig. 2, B) which we used as a non-haemolytic
2 control.

3 Enzymatic activities in the different purification fractions were tested by measuring 19 different
4 enzymes in the culture filtrate, the permeate P₁ and the final filtrate F₁ of both fungi strains.
5 Enzymatic activities were only detected in the crude culture filtrate, but not in the subsequent
6 purification fractions (Tab. 2).

7 To evaluate the specificity of the partially purified haemolysin, we tested sensitivity of erythrocytes
8 from different mammalian species, including cattle, sheep, swine, horses, dogs, and humans, to
9 the haemolysin. Sensitivity was highest of erythrocytes from equines, followed by erythrocytes
10 from sheep, cattle, swine, dogs, and humans (Fig. 3). As expected, none of the erythrocytes was
11 lysed by the filtrate F₁ from *T. verrucosum* var. *ochraceum*.

12 In a second experiment, we tested different eucaryotic cell lines from different species, including
13 cattle (MDBK), swine (ST), dogs (MDCK), human (HEp-2), and mouse (J774) cells, in their
14 sensitivity to cytolytic activities of the haemolysin. None of the tested cells showed any significant
15 membrane damage after treatment with different concentrations of the haemolysin, as tested by a
16 standard lactate dehydrogenase release assay (data not shown).

17 Further characterization of the haemolysin was done by testing stability against proteases and
18 temperature. Treatment of filtrate F₁ with proteinase K over night at 37 °C did not affect haemolytic
19 activity, and neither did heating at 100 °C for 10 min, freezing at – 20 °C for 24 h, and storage at
20 4 °C for 6 month.

21 **Discussion**

24 Dermatophytes are highly specialized keratinophilic fungi capable of inducing typical skin
25 infections in humans and animals. During the infection, the vegetative growth of hyphae occurs
26 within the stratum corneum of the epidermis by producing substances that diffuse through the
27 horny layer (Weitzmann and Summerbell, 1995). The pathological reactions observed in
28 dermatophytosis are mediated by constitutive and inducible hydrolytic enzymes such as different

keratinases, which have been speculated to be the most important dermatophyte virulence factor (Raubitschek, 1961). Furthermore, haemolytic activities in dermatophytes have been shown to correlate with severity and chronicity of clinical infections (López-Martínez et al., 1994). Biological membranes are of particular functional importance for an intact antimicrobial barrier, and this role makes them an interesting target for microbial damage (Salyers and Witt, 1994). We used erythrocytes from different mammalian species in an experimental model to study membrane damage, since erythrocytes are easily available, the structure and composition of their membranes are well known (Zwaal et al., 1973), and membrane destruction can readily be detected by visible haemolysis. In a qualitative haemolytic activity assay we could confirm haemolytic activity as previously described (Schaufuss and Steller, 2003), i.e. the colony of *T. mentagrophytes* was surrounded by a strong zone of complete haemolysis, whereas no haemolytic activity was seen in *T. verrucosum* var. *ochraceum*. Susceptibility of intact erythrocytes from different mammalian species to lysis was estimated by titration in a quantitative assay. This assay is an indirect test since the amount of haemolytic molecules is detected by measuring the absorbance of haemoglobin released from erythrocytes. The haemolysin from *T. mentagrophytes*, like many other haemolysins, exhibited species cell specificity. Of a broad variety of mammalian erythrocytes lysed by the toxin, horse erythrocytes were the most sensitive cells, thus horse erythrocytes required the smallest amount of toxin for lysis (Fig. 3). This is remarkable since only few of the haemolysins described so far are able to lyse horse erythrocytes (Kreger et al., 1971; Turner and Pickard, 1980). On the other hand, human erythrocytes were the most resistant cells, since the highest amount of toxin was required for lysis (Fig. 3).

The skin barrier is formed during epidermal differentiation, which results in stratum corneum (SC), composed of corneocytes and extracellular lipids forming a multilamellar membrane unit structure (Proksch et al., 2003). As a consequence of alterations in barrier function, fungi allergens can penetrate the skin more easily, thereby producing inflammable reactions (Woodfolk, 2005). Several different approaches were developed to study the penetration of molecules through SC like egg shell membranes (Washitake et al., 1980), or cellulose acetate membranes (Barry and El Eini, 1975). We used such a membrane placed directly on the surface of Columbia blood agar

plates for growth and release of haemolytic molecules by *T. mentagrophytes* (Fig. 1). This model suggests that haemolysins may also be able to penetrate the skin barrier and, therefore, may affect the outcome of dermatophyte infections. Though the haemolysin showed activity only against erythrocytes, but not other (tested) eukaryotic cells, it may play a role in pathogenesis in that it mediates penetration of the skin barrier by lysing the multilamellar membrane unit structure. However, this remains to be elucidated in future studies by experimental infections of the skin. Our approach to obtain highly purified haemolysin was not successful. However, we were able to partially purify the toxin using a combination of different filtration systems. This procedure resulted in a fraction of components smaller than 3 kDa, which were free of (detectable) enzymatic activity with respect to 19 tested enzymes. Since haemolytic activity in this fraction was enriched 2.6-fold, this showed that haemolysis was not due to any enzymatic activity of (known) fungal enzymes. Furthermore, the haemolysin was very stable against proteases and high temperature. Based on these results, we propose that the haemolysin secreted by *T. mentagrophytes* during growth is not an enzyme, but a very small and stable peptide. Such haemolytic peptides are known from other organisms, e.g. melittin, the main component of the venom of *Apis mellifera* (MW 2.84 kDa), or the alamethicin of *Trichoderma viride*, which comprises only 20 amino acids (Bernheimer and Rudy, 1986). These peptides interact with the lipid bilayer forming aqueous channels which span the membrane. Future studies will clearing up whether the haemolysin of *T. mentagrophytes* is structurally and functionally related to these peptides. This will surely contribute to our understanding of the molecular mechanisms of pathogenesis of dermatophyte infections.

Conclusion

Skin infections caused by dermatophyte fungi of the genera *Trichophyton*, *Epidermophyton* and *Microsporum* are emerging in recent years (Burzykowski et al., 2003). The etiologic role of *Trichophyton* infection in the development of allergic (Woodfolk, 2005) and skin infections (Weitzman and Summerbell, 1995) is well documented. On the other hand, the mechanism of pathogenicity and inflammatory responses are only poorly understood. The membrane damaging

1 potential of a haemolysin, such as the one described in this study, could play an important role
2 during skin infections. Analyses of the biochemical nature of this toxin will be necessary and its
3 role in skin infections should be elucidated.

4

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Legends to figures

Fig. 1: Haemolytic activity of *T. mentagrophytes* after growth on horse blood agar.

(A) *T. mentagrophytes* strain cbs 388.58. The colony is surrounded by a strong zone of complete haemolysis. (B) *T. verrucosum* var. *ochraceum* cultivated on the same agar shows no haemolytic activities surround the colony. Haemolysis after cultivation of *T. mentagrophytes* strain cbs 388.58 on cellulose acetate filter before (C) and after (D) removal of the filter membrane.

Fig. 2: Separation of different purification fractions by SDS-PAGE.

Fractions as indicated on top of the gels were separated by SDS-PAGE and subsequent silver staining. Fractions from culture filtrates of *T. mentagrophytes* (A) and *T. verrucosum* var. *ochraceum* (B) are shown. Molecular weight standard (in kDa) is shown on the left side.

Fig. 3 Haemolytic activities from the F₁ filtrate fraction based on the haemolysin of *T. mentagrophytes* against erythrocytes from indicated mammalian species. Activity was highest against those from horses, followed by erythrocytes from sheep, cattle, swine, dogs, and humans.

Preparation	PH	Protein [mg/ml]	Haemolytic activity (%)*	Specific activity (% / mg)**	Purification (-fold)
Culture filtrate	6.9	2.4	93	172	1
Permeate P₁	7.0	1.5	89	264	1.5
Filtrate F₁	7.0	1.0	100	444	2.6

1

2 **Table1:** Purification and enrichment of haemolytic activity in the supernatant of *T.*
3 *mentagrophytes*. *Results were expressed as % haemolysis (haemolytic activity) as
4 compared to haemolysis by ddH₂O. **Specific activity refers to haemolytic activity per mg
5 protein and was used to document enrichment of the haemolytic peptide.

Enzyme		Reactions					
Number	Enzymes	Culture filtrate		Permeate P ₁		Filtrate F ₁	
		<i>T. ment.</i>	<i>T. ver. v. ochra.</i>	<i>T. ment.</i>	<i>T. ver. v. ochra.</i>	<i>T. ment.</i>	<i>T. ver. v. ochra.</i>
1	Control	-	-	Complete negativ	Complete negativ	Complete negativ	Complete negativ
2	Alkaline phosphatase	(+)	+				
3	Esterase (C4)	+	+				
4	Esterase Lipase (C8)	+	+				
5	Lipase (C14)	-	-				
6	Leucine arylamidase	+	+				
7	Valine arylamidase	-	-				
8	Cystine arylamidase	-	-				
9	Trypsin	-	-				
10	α -chymotrypsin	-	-				
11	Acid phosphatase	+	+				
12	Naphtol-AS-BI-phosphohydrolase	-	-				
13	α -galactosidase	-	-				
14	β -galactosidase	-	-				
15	β -glucoronidase	-	-				
16	α -glucosidase	-	-				
17	β -glucosidase	+	-				
18	N-acetyl- β -glucosaminidase	-	+				
19	α -mannosidase	(+)	+				
20	α -fucosidase	-	-				

Table2: Testing for 19 enzymes in different preparations indicating activities only in the culture filtrate.

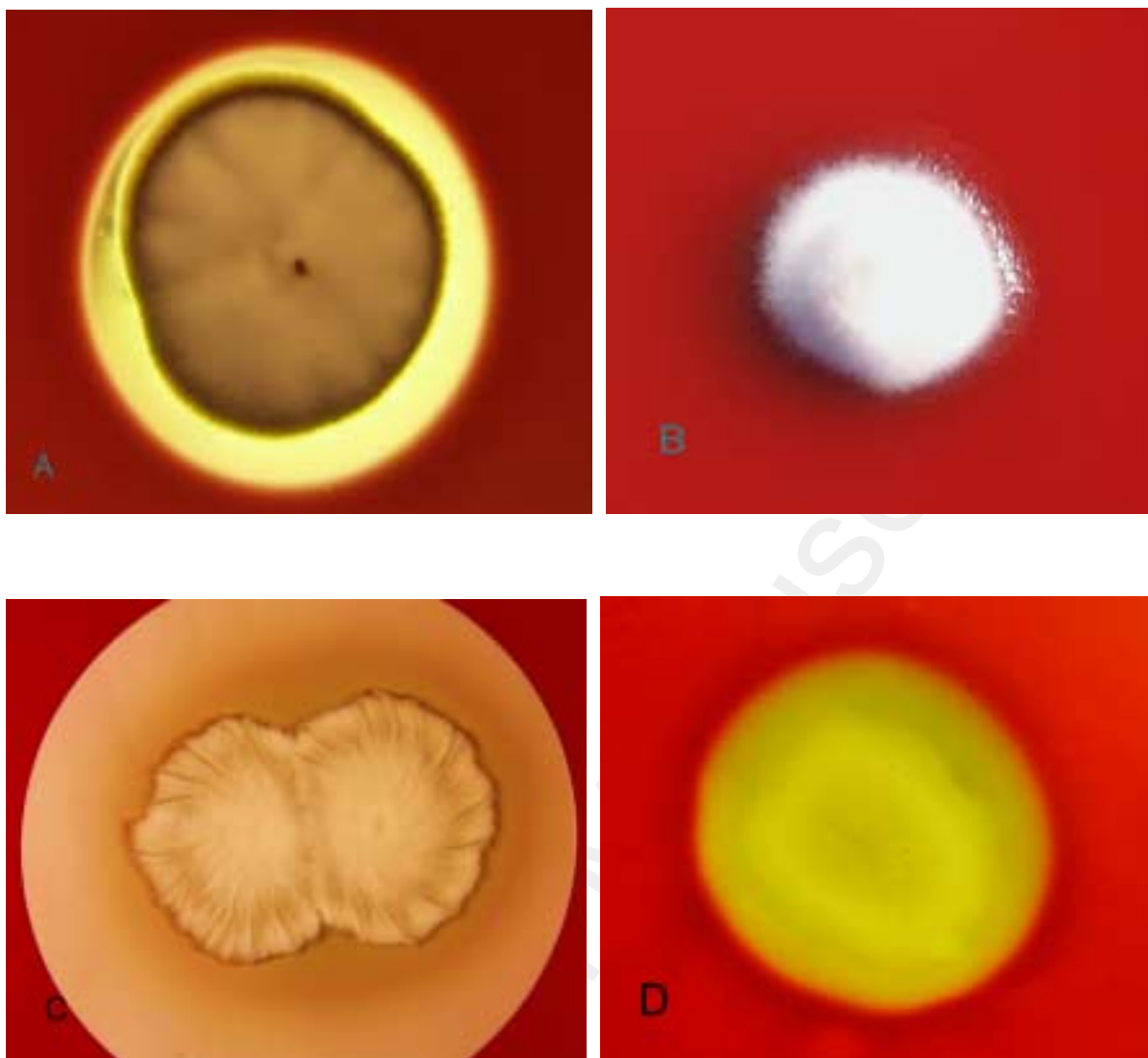


Fig. 1

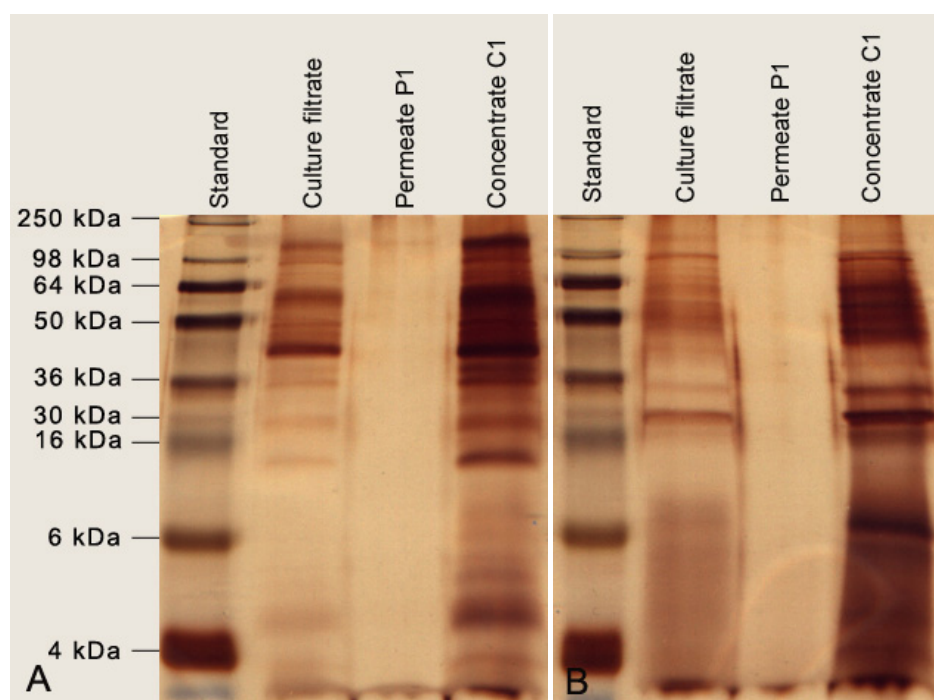


Fig. 2

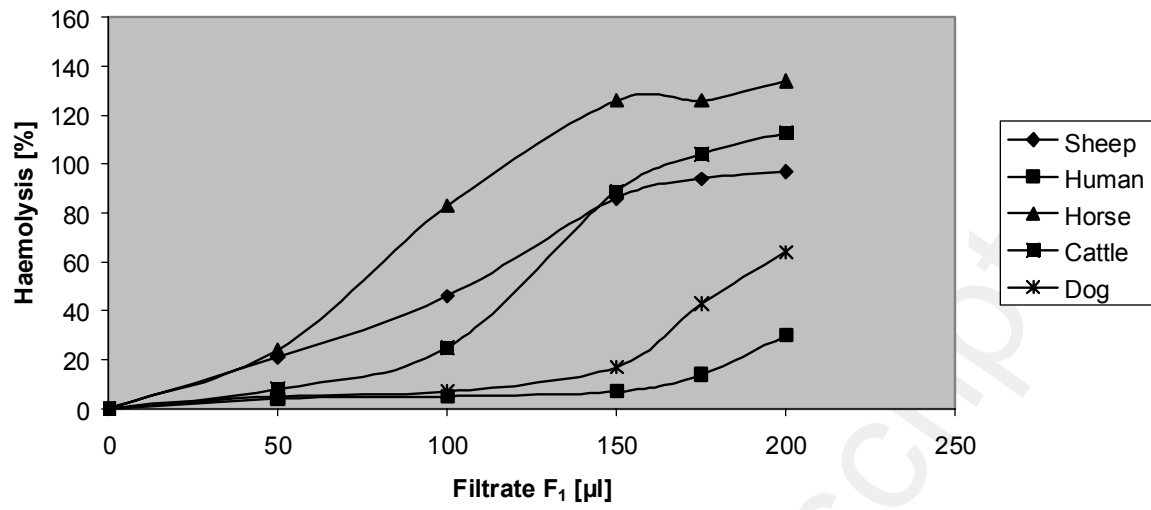


Fig. 3