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1 **Isolation and characterization of a haemolysin from**

2 ***Trichophyton mentagrophytes***

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1 **Abstract**

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

20

21 **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 cytolysins 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*.

1 **Materials and Methods**

3 **Strains**

4 *Trichophyton mentagrophytes* strain cbs 388.58 (Centraalbureau voor Schimmelcultures, Utrecht,
5 the Netherlands), isolated from a dog, and *T. verrucosum* var. *ochraceum* strain SM117
6 (Serumwerk Memsen, Hoyerhagen, Germany), isolated from a calve, were used in this study. The
7 latter was used as negative control in the haemolytic activity assays. The strains were grown on
8 Sabouraud agar containing 4% glucose (Oxoid CM 41; Unipath, Basingstoke, U.K.) for 15 days at
9 27°C, and controlled for purity and identity as described previously (Schaufuss and Steller, 2003).

11 **Qualitative haemolytic activity assay**

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

19 **Quantitative haemolytic activity assay**

20 Sheep erythrocytes were isolated and purified from 50 ml freshly drawn blood by centrifugation at
21 1200 x g for 10 min at 4 °C and subsequent washings with saline until the supernatant was clear,
22 and were then resuspended in saline to a final concentration of 3 % (w/v). Titrations were run in V-
23 form 96-well microtiter plates (Sarstedt, Newton, USA), starting with 225 µl of the samples to be
24 tested in 25 µl dilution steps to a final volume of 50 µl. Each dilution was filled up to a volume of
25 250 µl with saline, and subsequently 50 µl of a 3% suspension of washed erythrocytes were
26 added. The plates were incubated at 37 °C for 2 h. Non-lysed erythrocytes were removed by
27 centrifugation at 1000 x g for 5 min. The supernatant was transferred to a flat-bottom, 96-well
28 microtiter plate for spectrophotometric measurements at $A_{550\text{ nm}}$. In controls, samples to be tested

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

5 **Filter test**

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

12 **Partial purification of the haemolysin from culture filtrate**

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

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

1 **SDS-PAGE**

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

8 **Determination of enzymatic activities**

9 Culture filtrates, permeates P₁ and filtrate F₁ were also examined semi-quantitatively for the
10 presence of different enzymes using the API-ZYM system (API ZYM, Biomérieux, Hazelwood,
11 USA) as described by Brasch and Zaldua, (1994).

13 **Specificity of haemolytic activity**

14 Specificity of haemolytic activity (using erythrocytes from various species, including sheep, cattle,
15 swine, horses, dogs, and humans), were determined using filtrate F₁ in the quantitative haemolytic
16 activity assay described above. Erythrocytes were isolated and purified from freshly drawn blood
17 from respective animal species, which were kindly provided by our Clinical departments. After
18 washing, the erythrocyte suspensions were adjusted to a concentration of 3 % (w/v) as described
19 above.

20 In addition, the cytolytic activity of the filtrate F₁ was determined using the lactate dehydrogenase
21 (LDH) release assay (Cytotoxicity 96[®] Assay; Promega, Heidelberg, Germany) according to the
22 manufacturer's instructions. For this, different cell lines, including HEp-2, J774, MDBK, MDCK,
23 and ST cells, were tested after incubation with 1:2, 1:3, 1:6 dilutions of the filtrate made in cell
24 culture media with a final volume of 150 µl for 3 hours. Cells were cultivated in DMEM (MDCK in
25 EMEM, MDBK in EDulb) (Gibco-Invitrogen, Heidelberg, Germany) supplemented with 10 % fetal
26 calf serum (FCS) (MDBK with 5 % FCS) and 5mM glutamine. Cells were seeded on 24-well plates
27 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 12 **Results**

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

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

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

22 **Discussion**

23

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

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

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

1 plates for growth and release of haemolytic molecules by *T. mentagrophytes* (Fig. 1). This model
2 suggests that haemolysins may also be able to penetrate the skin barrier and, therefore, may
3 affect the outcome of dermatophyte infections. Though the haemolysin showed activity only
4 against erythrocytes, but not other (tested) eukaryotic cells, it may play a role in pathogenesis in
5 that it mediates penetration of the skin barrier by lysing the multilamellar membrane unit structure.
6 However, this remains to be elucidated in future studies by experimental infections of the skin.
7 Our approach to obtain highly purified haemolysin was not successful. However, we were able to
8 partially purify the toxin using a combination of different filtration systems. This procedure resulted
9 in a fraction of components smaller than 3 kDa, which were free of (detectable) enzymatic activity
10 with respect to 19 tested enzymes. Since haemolytic activity in this fraction was enriched 2.6-fold,
11 this showed that haemolysis was not due to any enzymatic activity of (known) fungal enzymes.
12 Furthermore, the haemolysin was very stable against proteases and high temperature. Based on
13 these results, we propose that the haemolysin secreted by *T. mentagrophytes* during growth is not
14 an enzyme, but a very small and stable peptide. Such haemolytic peptides are known from other
15 organisms, e.g. melittin, the main component of the venom of *Apis mellifera* (MW 2.84 kDa), or the
16 alamethicin of *Trichoderma viride*, which comprises only 20 amino acids (Bernheimer and Rudy,
17 1986). These peptides interact with the lipid bilayer forming aqueous channels which span the
18 membrane. Future studies will clearing up whether the haemolysin of *T. mentagrophytes* is
19 structurally and functionally related to these peptides. This will surely contribute to our
20 understanding of the molecular mechanisms of pathogenesis of dermatophyte infections.

21

22 **Conclusion**

23

24 Skin infections caused by dermatophyte fungi of the genera *Trichophyton*, *Epidermophyton* and
25 *Microsporum* are emerging in recent years (Burzykowski et al., 2003). The etiologic role of
26 *Trichophyton* infection in the development of allergic (Woodfolk, 2005) and skin infections
27 (Weitzman and Summerbell, 1995) is well documented. On the other hand, the mechanism of
28 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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1 **Legends to figures**

2
3 **Fig. 1:** Haemolytic activity of *T. mentagrophytes* after growth on horse blood agar.

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

8
9 **Fig. 2:** Separation of different purification fractions by SDS-PAGE.

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

13
14 **Fig. 3** Haemolytic activities from the F₁ filtrate fraction based on the haemolysin of *T.*
15 *mentagrophytes* against erythrocytes from indicated mammalian species. Activity was highest
16 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	-	-				
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	-	-	Complete negativ	Complete negativ	Complete negativ	Complete negativ
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	-	-				

1

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

4

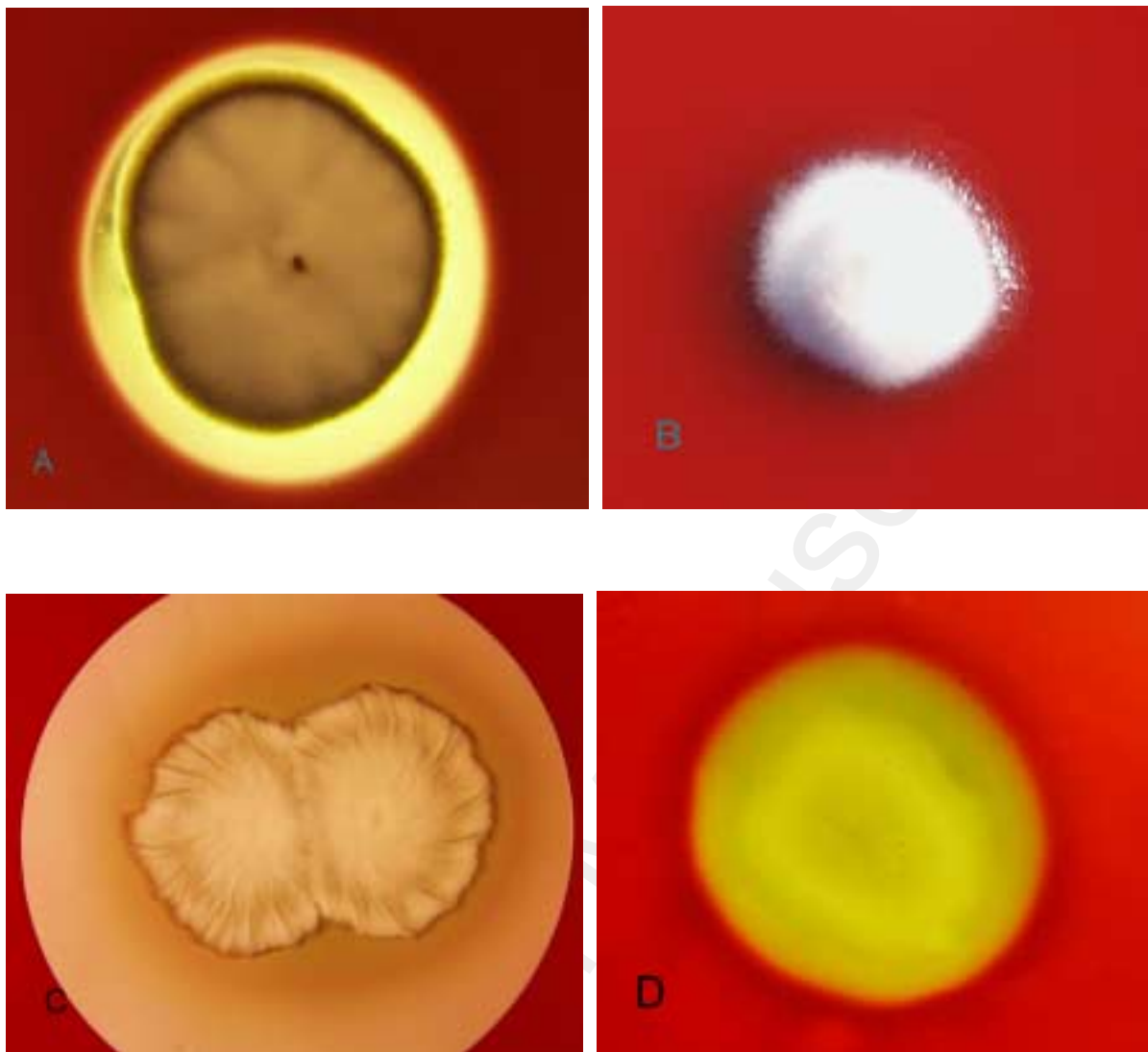


Fig. 1

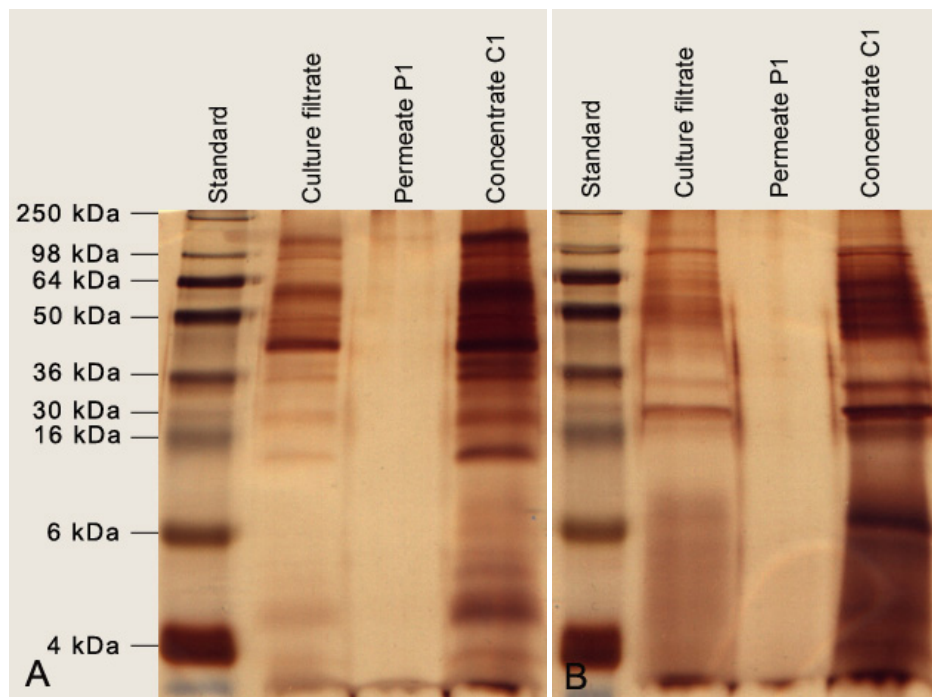


Fig. 2

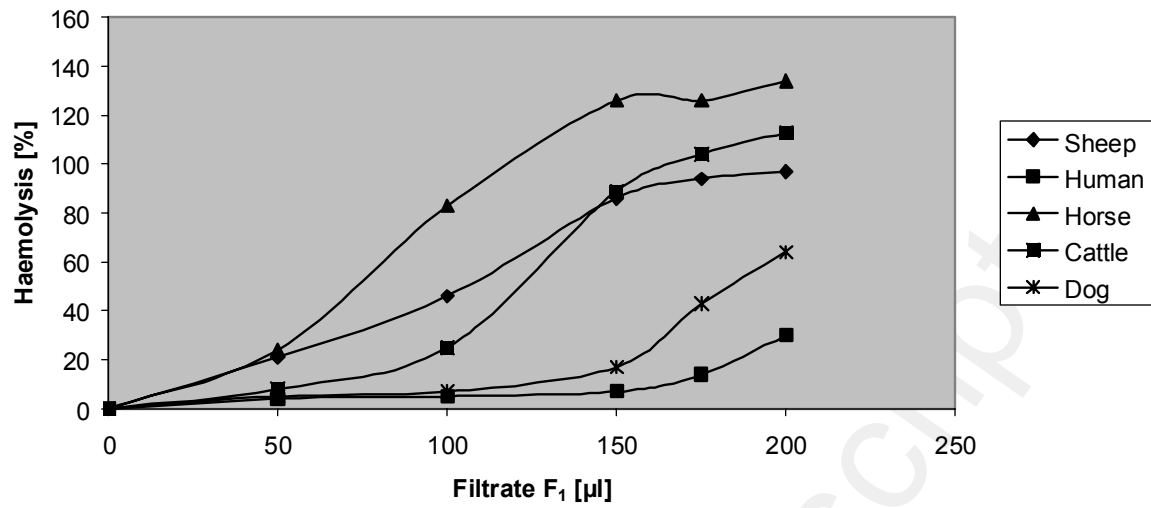


Fig. 3