

Detection of β -Glucuronidase Activity within *Actinomadura madurae* Grains of Human Actinomycetoma

Alejandro Palma-Ramos¹, Samantha Reyes-Mayén¹, Laura Estela Castrillón-Rivera¹,
Silvia Elena Fernández-López¹, Carmen Padilla-Desgarennes², María Elisa Vega-Memije³,
Roberto Arenas-Guzmán⁴, María Elisa Drago-Serrano¹, Teresita Sainz-Espuñes^{1*}

¹Department of Biological Systems, Universidad Autónoma Metropolitana-Xochimilco, Mexico City, México

²Dermatology Center “Dr. Ladislao de la Pascua”, Mycology Service, Mexico City Health Secretary, Mexico City, México

³General Hospital “Manuel Gea González”, Dermatology Service, Mexico City Health Secretary, Mexico City, México

⁴General Hospital “Manuel Gea González”, Mycology Service, Mexico City Health Secretary, Mexico City, México

Email: *trsainz@correo.xoc.uam.mx

Received 24 September 2013; revised 30 October 2013; accepted 30 November 2013

Copyright © 2014 by authors and Scientific Research Publishing Inc.

This work is licensed under the Creative Commons Attribution International License (CC BY).

<http://creativecommons.org/licenses/by/4.0/>



Open Access

Abstract

Actinomycetoma syndrome by *Actinomadura (A.) madurae* is characterized by a subcutaneous chronic lesion that affects fascia, muscle and bone. *A. madurae* produces colonies that form grains of less than 1 mm in diameter. Grains are surrounded and infiltrated by neutrophils involved in the grain disruption by enzymes like β -glucuronidase released after the neutrophil degranulation. The aim of this work was to evaluate the polysaccharide degradation of grains treated with β -glucuronidase and to detect the presence and activity of β -glucuronidase within the *A. madurae* grains. *Actinomadura madurae* grains from patients infected were processed to quantify the total content of polysaccharide with the phenol-sulfuric acid reaction. Grains were treated with β -glucuronidase at different conditions to evaluate the optimal polysaccharide degradation. Grains were analyzed to detect the enzyme by using anti-human β -glucuronidase antibody while enzymatic activity was assessed by evaluating the release of reduced sugars and by *in situ* enzymatic activity. Optimal degradation of polysaccharide in the grains treated with β -glucuronidase was found with 300 units/ml of enzyme and 24 hr of incubation at 37°C. Presence and activity of β -glucuronidase enzyme within the grains were detected. Results suggested that β -glucuronidase present within *A. madurae* grain resulted from degranulated neutrophils surrounding and/or infiltrated within the grain.

*Corresponding author.

Keywords

Actinomycetoma, *A. madurae*, β -Glucuronidase

1. Introduction

Actinomycetoma is a subcutaneous chronic infection from bacterial origin caused by *Actinomadura* (*A.*) *madurae* present among the population of some areas of developing countries like Mexico. The total number of actinomycetoma cases recorded at Mycology laboratory of Ladislao De La Pascua dermatological Center of Mexico City in 42 years (1956-1998) was 732. Seventy one (9.6%) corresponded to *A. madurae* affecting mainly females as the predominant gender. In México the distribution of actinomycetoma cases is located in three main areas: the Central Western, Southern Center and West of Hidalgo state [1].

Actinomycetoma is a syndrome characterized by disfiguring, painless inflammatory lesions and fistulas that affect the cutaneous and subcutaneous tissue, fascia and bone. Actinomycetoma is a suppurative infection which usually affects the lower extremities, but it can occur in almost any region of the body. Actinomycetoma evolution is very slow and therefore the inability condition is lately achieved (months or years) [2] [3].

Actinomadura madurae produces white and soft grains with a diameter size of 1 - 10 mm and by direct examination it can be observed that the grains are surrounded by swaths of long fringes, consisting of filaments, often bifurcated and uneven edges. Histopathological observation of tissue samples stained with hematoxylin-eosine shows multilobular grains with irregular or uneven edges and with a blue peripheral band intensely stained, while the center is often devoid of color [1]-[3].

Hematoxylin-eosin staining of *A. madurae* actinomycetoma has evidenced neutrophil infiltration, as well as disruption of the surface of *A. madurae* grain [4]. This may reflect the presence of enzymes released by the neutrophil degranulation.

Neutrophils are endowed with azurophil (primary) granules containing polysaccharide degrading enzymes like β -glucuronidase released in surrounding environment after degranulation [5].

Polysaccharides are commonly components of a matrix where the bacteria are embedded to generate biofilms which functions as a shield of protection that decreases the antimicrobial efficiency of antibiotics [6] [7].

Since β -glucuronidase enzyme released by neutrophils may degrade polysaccharide components of biofilm within *A. madurae* grain the aim of this work was 1) to evaluate polysaccharide degradation of *A. madurae* grain in presence of β -glucuronidase enzyme, 2) to detect the presence and 3) the enzymatic activity of β -glucuronidase released by neutrophils within the grain. This approach may impact on the developing of an auxiliary treatment toward *A. madurae* actinomycetoma due to the enzymatic degradation of polysaccharide biofilm by β -glucuronidase that eventually would allow the entry of antibiotics to eliminate the *A. madurae* inside the grain.

2. Materials and Methods

2.1. Actinomycetoma Grain Sampling

Actinomycetoma grains from two different patients with positive diagnostic for actinomycetoma by *Actinomadura madurae* were obtained either at the Mycology laboratory of "Ladislao De La Pascua" Dermatological Centre or at Mycology laboratory of "Manuel Gea González" General Hospital. Patients decided voluntarily participate therefore they signed a written informed consent form approved by the ethic commission for biomedical research in human according to the guidelines from the Secretary of Health, prior to the onset of the study.

2.2. Polysaccharides Quantification in *Actinomadura madurae* Grains

Prior to treatment with β -glucuronidase, total polysaccharide content in the actinomycetoma grains was quantified using the method of phenol-sulphuric acid reaction [8]. Two samples with 4 beads each, were dried, weighed and grounded. Afterwards, samples were suspended in 1 ml of distilled water and then 3 ml of sulfuric acid (H_2SO_4 , 95.5% w/v, specific gravity 1.84) and 50 μ l of phenol (80% w/v) were added. The absorbance value of the colorful compounds produced were measured spectrophotometrically at 485 nm to quantify the total content of polysaccharide based on a standard curve by plotting the concentration of glucose (2 to 20 μ g/ml) vs. the cor-

responding absorbance values.

2.3. “*In Vitro*” Determination of the Polysaccharide Degradation of *Actinomadura madurae* Grain with the β -Glucuronidase Enzyme

In order to determine the optimal time of incubation, 3 grains were used for this purpose. Each whole grain was suspended in 1 ml of sodium acetate buffer (100 mM, pH 3.8), inside a well from a 24 well culture plate and 100 μ l of commercial β -glucuronidase (EC 3.2.1.31 Sigma-Aldrich G025) solution at a concentration of 900 units/ml was added. Each whole sample grain was then incubated for 24, 48 or 72 hours in a humid chamber at 37°C and 150 rpm agitation.

To test the optimal concentration of the enzymatic activity, 3 sample grains were treated each with 300, 600 or 900 units/ml of β -glucuronidase for 24 h under the same conditions of incubation in a humid chamber at 37°C and 150 rpm agitation [9] [10].

After incubation, samples were centrifuged and the supernatants were collected to estimate the content of the total degraded polysaccharides according to the method of Dubois described earlier [8].

An additional assay was done to visualize the enzymatic degradation of the grain by using a concentration of 300 units/ml β -glucuronidase and incubation time of 24 h. One untreated grain was used as control.

2.4. *In Vitro* Determination of β -Glucuronidase Activity within Grains

The activity of the β -glucuronidase secreted by PMN infiltrated within the grain was analyzed. The *in vitro* enzyme activity of β -glucuronidase on *A. madurae* grains, was performed by using phenolphthalein-glucuronic acid as the substrate, so when the reaction takes place the enzyme frees phenolphthalein, which is then quantified by spectrophotometry at 540 nm [11].

To do so, the grain was finely sectioned with a scalpel and the obtained fragments were suspended in 700 μ l of 100 mM sodium acetate buffer, pH 3.8 and pre-warmed at 37°C. After that, 700 μ l of 3 mM phenolphthalein-glucuronic acid substrate was added and the mixture was incubated 30 min with agitation at 150 rpm. The reaction was stopped by addition of 5 ml of 200 mM glycine buffer pH 10.4, and the absorbance at 540 nm was determined.

In order to quantify the amount of phenolphthalein released in the above reaction, a phenolphthalein standard curve was assayed using the following concentrations: 12.3, 30.7, 61.4, 92.1, 153.6 and 307.2 μ g/ml and the absorbance at 540 nm were determined.

2.5. *In Situ* Detection of β -Glucuronidase in *A. madurae* Grains

Presence of β -glucuronidase was detected by using an anti-human β -glucuronidase antibody the antigen-antibody interaction was revealed with a commercial kit (Cell and Tissue Staining Kit, Goat Kit, HRP-AEC System by R&D (Minneapolis, Mn, USA Cat No. CTS009).

2.6. *In Situ* β -Glucuronidase Activity Detection in *A. madurae* Grains

In addition, histological sections were used in order to detect the activity *in situ* of β -glucuronidase within the grains according to instructions of the commercial kit of lymphocyte enzymes (Sigma Aldrich 181C-1KT, Lot.060M4351, Germany). The kit used naphthol AS-BI, glucuronic β -D-acid as the substrate and pararosaniline as an indicator. When the substrate takes contact with the enzyme (β -glucuronidase) present in the histological cuts forms a red chromogenic complex with the naphthol-pararosaniline and it may be seen under the microscope. Methylene blue was used as contrast dye. The test was done in 4 histological sections [12].

3. Results

3.1. “*In Vitro*” Determination of the Enzyme Required for the Polysaccharide Degradation

Enzymatic activity of β -glucuronidase was tested at different times of incubation in order to assess the optimal time required for the enzymatic degradation of polysaccharide on the grain. **Table 1** showed that 24 h was optimal time for the polysaccharide degradation by the β -glucuronidase enzyme using 900 units/ml.

Once the optimal incubation time was determined, the optimal concentration of the enzyme was assessed. **Table 2** showed that 300 units/ml rendered the maximum sugar release.

3.2. Enzymatic Activity of Commercial β -Glucuronidase Added to the Grains

This analysis using 300 units /ml of the enzyme incubated during 24 h, was made to visualize the effect of β -glucuronidase in the actinomycetome treated grains. By comparison with the untreated grain (**Figure 1(a)**) a substantial degradation of the exopolysaccharide in the grain surface was observed (**Figure 1(b)**). Enzymatic degradation was observed also in supernatants obtained from the grains (**Figure 1(c)**). These results showed the ability of β -glucuronidase for the exopolysaccharide degradation.

3.3. Quantitative Evaluation of the Effect of β -Glucuronidase Released by Polymorphonuclear Neutrophils (PMN) Infiltrated into *Actinomadura madurae* Grain

Results in **Table 3** showed the standard curve of phenolphthalein for the quantification of the activity of the enzyme within the grain using a commercial enzyme. It also showed the value of the activity of the enzyme within the grain sample. The sample presented an absorbance of 0.030 which correspond to the 51.34 $\mu\text{g/mL}$ of phenolphthalein released. According to the reaction between the enzyme and the substrate the calculated activity was 30.78 units/mL of β -glucuronidase released by PMN infiltrated in the grain sample.

3.4. *In Situ* Detection of β -Glucuronidase Enzyme within *A. madurae* Grain

As shown in **Figure 2**, the presence of the enzyme was detected by the appearance of dots of red color within the *A. madurae* grain using anti-human β -glucuronidase antibody.

Table 1. Enzymatic degradation of polysaccharide present in the grains of *Actinomadura madurae* at different incubation times with 900 units/ml of β -glucuronidase.

Weight sample (μg)	Total polysaccharide content ($\mu\text{g/ml}$)	Total % polysaccharide/weight sample	Incubation time (h)	Content of reduced sugar in supernatant ($\mu\text{g/ml}$)	% polysaccharide degraded
200	23.9	11.95	24	7.36	31.32
200	24.7	12.35	48	3.40	13.77
400	50.5	12.62	72	3.36	6.67

Table 2. Enzymatic degradation of the polysaccharide present in the grain of *Actinomadura madurae* at different concentrations of β -glucuronidase.

Sample (μg approx.)	Enzyme [units/ml]	Polysaccharide in supernatant [$\mu\text{g/ml}$]	% polysaccharide degraded
200	900	10.0	28.0
200	600	5.34	9.62
200	300	2.64	4.76

Table 3. Standard curve of phenolphthalein using a commercial enzyme for the analysis of the enzymatic activity of the β -glucuronidase from PMN infiltrated into *Actinomadura madurae* grain using 3 mM phenolphthalein-glucuronic acid substrate.

Tube	Absorbance	Concentration of phenolphthalein ($\mu\text{g/mL}$)
1.	0.0058	12.3
2.	0.0142	30.72
3.	0.0359	61.44
4.	0.0509	92.16
5.	0.0925	153.6
6.	0.179	307.2
Sample problem (grain)	0.030	51.34

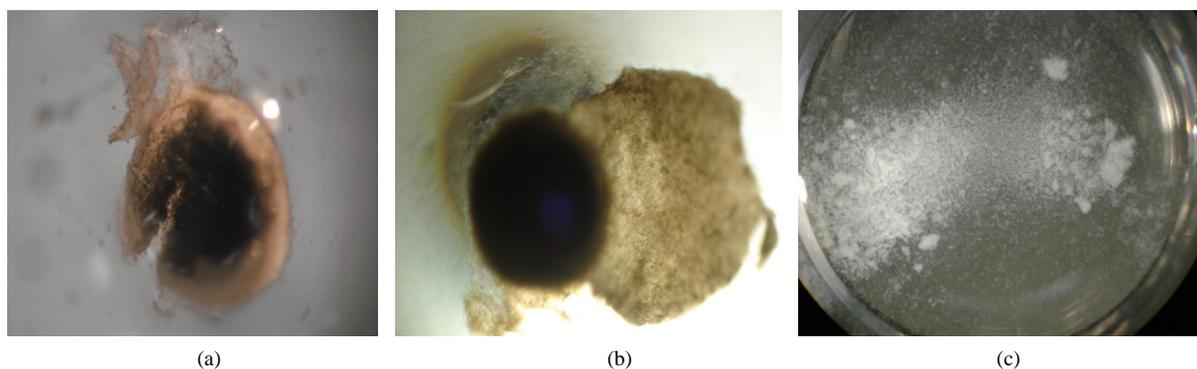


Figure 1. (a) Untreated *Actinomadura madurae* grain; (b) *Actinomadura madurae* grain treated with β -glucuronidase; (c) Supernatant with residues from the treatment of grain with β -glucuronidase.

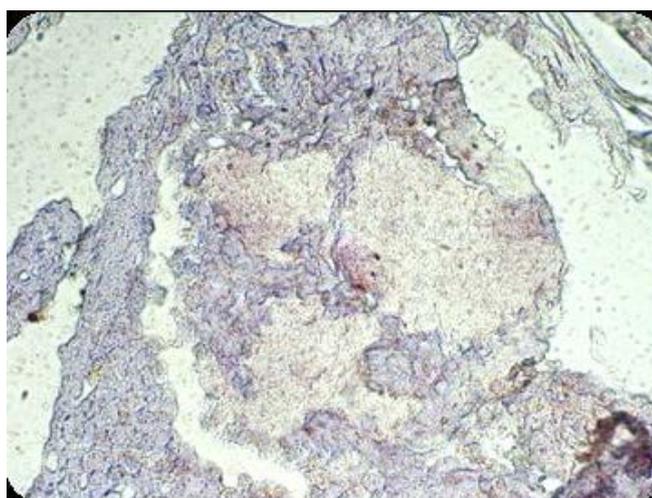


Figure 2. *In situ* detection of β -glucuronidase enzyme within *A. madurae* grain visualized by the presence of red-brown dots.

3.5. Histological Analysis

A qualitative analysis to detect the activity of the β -glucuronidase was made on histological sections of actinomycetomas from patients affected with *A. madurae* using a lymphocyte enzyme commercial kit. Presence of the enzyme activity was detected by a dim red coloration. Decreased red coloration suggests a limited enzymatic activity on the polysaccharide by β -glucuronidase released by PMN infiltrated into the actinomycetoma grain (Figure 3).

4. Discussion

Under conditions of 900 units/ml of β -glucuronidase and 24 hr of incubation at 37°C it was revealed that 30.32% of polysaccharide was substrate for enzymatic degradation (Table 1).

According to the results, less degradation of polysaccharide grain was found with 600 and 900 units/ml of β -glucuronidase suggesting an effect of saturation of the enzyme (Table 2). Twenty eight percent of total polysaccharide of *A. madurae* grains was degraded in the presence of β -glucuronidase under optimal conditions of 24 hrs and 300 unit/ml of enzyme (Table 2). This may indicate that some substrates degraded by β -glucuronidase may include acidic sugars like β -glucuronosides although the bulk of polysaccharides were not degraded by this enzyme. Other acidic substrates for β -glucuronidase activity may include exopolysaccharides as in the case of sulfated acid mucopolysaccharides detected in the *A. madurae* grain [13] [14]. It has been proposed that sulfated acid mucopolysaccharide is a sticky material of the biofilm matrix which acts as a glue for joining the bacteria inside the grain [14].

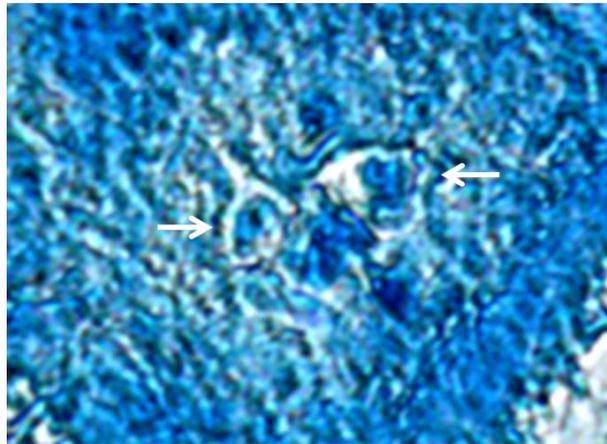


Figure 3. Positive activity of β -glucuronidase in histological sections, shown by the red coloration on the center of the grain of *Actinomadura madurae*.

In the current contribution we could detect the presence and the activity of β -glucuronidase inside *A. madurae* grain which probably contributed for 1) the disruption of the grain surface and for 2) the neutrophil infiltration inside the grain as evidenced previously by hematoxylin-eosin staining of *A. madurae* actinomycetoma from infected patients [4].

Underlying mechanism of infiltration of neutrophils in the skin lesion involve a complex web of ligand-receptor interactions of addressins expressed in neutrophil surface with their corresponding receptors in endothelial blood vessels and neutrophil receptors for chemotactic factors released by skin cells from the focus of lesion [15]. In the case of *A. madurae* mechanisms which account neutrophil trafficking inside grains are not fully known but may involve the release of tumor necrosis factor (TNF)- α and interleukin (IL)-1 β by keratinocytes as evidenced in biopsies of patients [4]. Proinflammatory cytokines like TNF- α and IL-1 β have been ascribed to neutrophil activation which contributes for degranulation and infiltration of neutrophils inside *A. madurae* grain [4].

In this study direct evaluation of enzymatic degradation of purified β -glucuronosides and/or sulfated acid polysaccharides by β -glucuronidase activity was not made, neither the characterization of other enzymes like heparanase from specific granules released by neutrophil. Evaluation of both events may provide a wide picture of the impact of neutrophil infiltration and degranulation within *A. madurae* grain. Upon activation, neutrophils release granule proteins and chromatin that together form extracellular fibers known as extracellular traps that bind to the pathogen surface [16]. Neutrophil extracellular traps have a key role in the innate immunity for killing of pathogens including bacteria, yeasts and fungi [16]. In spite of these limitations the evidences presented in the current contribution suggest that under *in vivo* conditions β -glucuronidase may contribute, along with other enzymes released *in situ* after the neutrophil degranulation, in the disruption of *A. madurae* lesion.

Host factors related with chronic infection of *actinomycetoma* have been ascribed to a depressed cellular immune response related with a decrease number of epidermal langerhans cells [17]. This chronic infection is aggravated because the presence of polysaccharides forming the biofilm matrix which shield and protect to the bacteria toward the action of antibiotics [18]. Along with other components present in primary and secondary neutrophil granules as lysozyme, lactoferrin and mieloperoxidase, β -glucuronidase may be used as an auxiliary for the antibiotic treatment.

References

- [1] Lavalle, A.P., Padilla, D.M., Pérez, G.J., Rivera, I. and Reynoso, R.S. (2000) Micetomas por *Actinomadura madurae* en México. *Revista del Centro Dermatológico Pascua*, **9**, 19-24.
- [2] Welsh, O., Vera-Cabrera, L., Welsh, E. and Salinas, M.C. (2012) Actinomycetoma and Advances in Its Treatment. *Clinics in Dermatology*, **30**, 372-381. <http://dx.doi.org/10.1016/j.clindermatol.2011.06.027>
- [3] Venkatswami, S., Sankarasubramanian, A. and Subramanyam, S. (2012) The Madura Foot: Looking Deep. *The International Journal of Lower Extremity Wounds*, **11**, 31-42. <http://dx.doi.org/10.1177/1534734612438549>

- [4] Palma, R.A., Castrillón, L.R., Encinas, M.G., Padilla, C.D. and Guzmán, R.A. (2009) Participación De Los Queratinocitos En La Respuesta Inmunitaria Contra El Actinomicetoma. *Dermatología Revista Mexicana*, **53**, 225-233.
- [5] Mollinedo, F. (2003) Human Neutrophil Granules and Exocytosis Molecular Control. *Inmunología*, **22**, 340-358.
- [6] Mah, T.F. (2012) Biofilm-Specific Antibiotic Resistance. *Future Microbiology*, **7**, 1061-1072. <http://dx.doi.org/10.2217/fmb.12.76>
- [7] Petrova, O.E. and Sauer, K. (2012) Sticky Situations: Key Components That Control Bacterial Surface Attachment. *Journal of Bacteriology*, **194**, 2413-2425. <http://dx.doi.org/10.1128/JB.00003-12>
- [8] Dubois, M., Gilles, K., Hamilton, J., Rebers, P. and Smith, F. (1956) Colorimetric Method for Determination of Sugars and Related Substances. *Analytical Chemistry*, **28**, 350-356. <http://dx.doi.org/10.1021/ac60111a017>
- [9] Goldstein, G. (1961) Serum B-Glucuronidase Assay by the Phenolphthalein Mono-B-Glucuronide Method. *Clinical Chemistry*, **7**, 136-142.
- [10] Fishman, W.H., Springer, B. and Brunettei, R. (1948) Application of an Improved Glucuronidase Assay Method to the Study of Human Blood Beta-Glucuronidase. *Journal of Biological Chemistry*, **173**, 449-456.
- [11] Talalay, P., Fishman, W.H. and Huggins, C. (1946) Chromogenic Substrates; Phenolphthalein Glucuronic Acid as Substrate for the Assay of Glucuronidase Activity. *Journal of Biological Chemistry*, **166**, 757-772.
- [12] Lorbacher, P., Yam, L. and Mitus, W. (1967) Cytochemical Demonstration of B-Glucuronidase Activity in Blood and Bone Marrow Cells. *Journal of Histochemistry & Cytochemistry*, **15**, 680-687.
- [13] Palma, R.A., Castrillón, L.R., Padilla, D.M. and Reyes, F.F. (2005) Caracterización Histoquímica De Micetomas Por Actinomadura Madurae, Nocardia Brasiliensis Y Madurella Mycetomatis. *Dermatología Revista Mexicana*, **49**, 51-58.
- [14] Palma, R.A., Castrillón, R.L., Padilla, D.M., Rosas, H.L. and Márquez, C. (2006) Purificación Y Determinación De La Estructura De Los Polisacáridos Que Forman El Cemento De Unión En Granos De Actinomicetomas Ocasionados Por Actinomadura Madurae Y Nocardia Brasiliensis. *Dermatología Revista Mexicana*, **50**, 165-173.
- [15] Luster, A.D., Alon, R. and Von Andrian, U.H. (2005) Immune Cell Migration in Inflammation: Present and Future Therapeutic Targets. *Nature Immunology*, **6**, 1182-1190. <http://dx.doi.org/10.1038/ni1275>
- [16] Brinkmann, V. and Zychlinsky, A. (2012) Neutrophil Extracellular Traps: Is Immunity the Second Function of Chromatin? *The Journal of Cell Biology*, **198**, 773-783. <http://dx.doi.org/10.1083/jcb.201203170>
- [17] Guimarães, C.C., Castro, L.G. and Sotto, M.N. (2003) Lymphocyte Subsets, Macrophages and Langerhans Cells in Actinomycetoma and Eumycetoma Tissue Reaction. *Acta Tropica*, **87**, 377-384. [http://dx.doi.org/10.1016/S0001-706X\(03\)00139-6](http://dx.doi.org/10.1016/S0001-706X(03)00139-6)
- [18] Castrillón, R.L., Palma, R.A. and Padilla, D.M. (2010) Importancia De Las Bio-Películas En La Práctica Médica. *Dermatología Revista Mexicana*, **54**, 14-24.