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IDENTIFICATION OF RHOPTRY PROTEINS (ROP5) VIRULENCE FACTORS OF MICROSPORIDIA AMONG APPARENTLY HEALTHY AND IMMUNOCOMPROMISED PATIENTS

Ubanwa, E. D.
Department of Applied Biology and Biotechnology, Faculty of Applied National Science, Enugu state University of Science and Technology.

Omalu, I. C. J.
Federal University of Technology Minna.

Njom, V. S.
Enugu State University of Science and Technology.

Abah, O. M.
National Examination Council

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Abstract: *The rhoptries are key secretory organelles from apicomplexan parasites that contain proteins involved in invasion and modulation of the host cell. Many rhoptry proteins have been shown to be key players in apicomplexan and virulence. Rhoptry 5 protein (ROP5) have been identified as important virulence factor uses by microsporidia species to invade the host. This ROP5 varies from difference diseases conditions from 0.021 Optical density to 0.411 Optical density, the minimum cut off level at which microsporidia becomes virulence was 0.261 OD. The highest OD was among Mal/AIDS disease condition 0.328 OD and the least was from TB + patients 0.236 OD. Age was also a determinant factor of the virulence as highest ROP5 was from 0 - 14 years 0.411 OD and least was from 20 - 24 years 0.212 OD. Understanding the mechanism of this factor will help to improve the lives of immunocompromised individual.*

Introduction

Microsporidia have been recognized as opportunistic pathogens of both immunocompitents and immunocompromised patients, that causes diseases called microsporidiosis (Lores, *et al.* 2012). Intracellular parasites have to contend with immune responses mounted by the hosts they infect if they are to survive long enough to effectively transmit. The parasite Microsporidia is one of the more successful parasites in terms of transmission as it can infect all mammals and many birds (Dubey and Jones, 2018), both of which serve as

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intermediate hosts where the parasite propagates asexually. Microsporidia readily infects humans but mainly causes disease in situations where the immune system has become compromised, although associations between parasite genotype and disease severity may also occur among apparently healthy individuals (Ubanwa *et al.*, 2018)

Microsporidia is a protozoan parasite belonging to the phylum Microspora that comprises various parasites responsible for many human and animal diseases known as Microsporidiosis. As an obligate intracellular parasite, Microsporidia actively invades host cells by an actin-myosin dependent mechanism (Ubanwa *et al.*, 2019) that also requires the coordinated exocytosis of proteins located in apical secretory organelles (Carruthers and Sibley 2017), namely the micronemes and rhoptries. Successful invasion proceeds through several distinct steps including apical attachment, formation of a moving junction, progression of the parasite through the junction and concomitant establishment of the parasitophorous vacuole within which the parasite further reside and replicate. Micronemal proteins are mostly adhesins secreted during invasion and then expressed at the surface of parasite and allow motility, recognition and attachment to the host cell through interactions with receptors expressed at the host cell surface (Lebrun *et al.*, 2007).

Rhoptries are club-shaped elongated organelles divided into two distinct suborganellar compartments, the bulbous part and the more anterior thin duct (or neck) through which rhoptry proteins are secreted (Dubremetz, 2017), however, no documented report on determinants responsible for maintaining this shape. Rhoptries biogenesis is driven by vesicular trafficking from the Golgi apparatus. This rhoptries are first detected as immature organelles, called pre-rhoptries, which are large vesicles containing a heterogenous dense material, located between the Golgi and the apical area of developing tachyzoites. As many rhoptry proteins have been shown to be key players as factor responsible Microsporidia invasion, replication and virulence, a better understanding of the optimal density of rhoptries protein concentration seems to be crucial hence this research.

Materials and Methods

Ethical Clearance, Participants Information and Consent Form

Ethical clearance was approved by Ethical committee Federal University of Technology and General Hospital Minna, Niger state, and all works were performed according to the guidelines for human experimental in clinical research stated by the Federal Ministry of Health Nigeria.

Samples collection

About 45 blood samples from comfirm positive patients of microsporidia infection were collected through intravenuous and discharge into EDTA bottles, stored under room temperature.

Separation of Serum from Blood

In a centrifuge capable of safely spinning blood tubes, spin the blood at roughly 2500 rpm for 10 minutes at room temperature. The tubes were removed from the centrifuge, and in a clean and safe environment, and opens to access the serum located at the top of the specimen. Using a sterile transfer pipet to transfer 2x 1.5 ml of serum into appropriately labeled screw-cap cryovial tube. As soon as possible, the serum was place and store at -80° C.

Preparation and Extraction of Antigen

Following 3 cycles of freezing - thawing, the spores were mixed with solid glass beads (1:3, Jencons (Scientific) Limited, UK), and soni-

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cated (30 min. 60 W). The number of spores before and after disruption was counted in a haematocylometer to ensure at least 95 per cent spore disruption in the homogenate.

Enzyme Linked Immunosorbent Assay (ELISA)

Polystyrene microtitre plates (Immuno plate Maxisorb; Nunc, Roskilde, Denmark) were coated for 3 days at 4°C with 150µl diluted antigen solution. The coating was done using Phosphate Buffer Saline (170 mM, pH 7.4), and distilled water. The plates were washed 3 times in Phosphate Buffer Saline (PBS), and some of them were incubated for Ih, with horse serum (100µl/ml), as blocking solution and non-fat dry milk (30 mg/ml).

All sera were tested in 4 wells, 2 wells coated with *E. binned* and *E. cuniculi* antigen and 2 wells coated with control. Thus, each 96-well miocrotitre plate harboured a maximum of 22 test sera positive and negative control (lebrum *et al*., 2015).

Virulence Markers (Rhoptry proteins)

Coating of Capture Antibody

The capture or coating antibody was appropriately dilute in carbonate-bicarbonate buffer (PBS). Capture antibodies were typically plated at 0.2 to 10 µg/ml. Affinity purified antibodies were prefer do to it active than IgG fraction. The diluted capture antibody was Pipette (0.2 ml) in each well of a microtiter plate and incubated (covered) for 1 hour at 37 °C. The coating solution were removed and wash 3 times with washing buffer (PBS – T)

Application of Samples and Control

About 0.2 ml of appropriately diluted samples and controls were added into the appropriate wells. Samples were diluted in PBS in the 10 ng-10 µg/well range (although the more sensitive the assay, the less sample is required). The plate was incubated at room temperature for 1hour. The sample and control were removd and wash 3 times with washing buffer (PBS – T).

Detection Antibody

The enzyme-labelled detection antibody was marked, 0.2 ml of the appropriately diluted detection antibody were pipetted to each well. The plate was incubated at room temperature for 30 minutes. The enzyme substrate was prepared immediately before use (all reagents were brought to room temperature before use). The plate was washed 3 times with washing buffer (PBS-T) and appropriate substrate solution were added, the plate was allowed to develop for 30 minutes in the dark. Coloure changes were observeded (Yellow or orange), 50 µl of stop solution were added per well (10% H_2S04) to each well. Absorbance was read directly in a microplate reader (MRX, Dynex Technologies, Inc., USA) at 402 nm /450 nm.

RESULTES

Rhoptries an apical secretory organelle involved in invasion, rhoptry 5 (ROP5) protein kinase have been identified as virulence factore uses by microsporidia with differents level in study population. The activity of microsporidia complex Rhoptry Kinase (ROP5) virulence in Apparently Healthy subjects and Immunocompromised patients from the assay represented by the graph spikes show the levels of rhoptry 5 protein in patients who were positive for microsporidiainfection. Each spike represents the level (concentration) of rhoptry 5 virulence in hosts, detected by direct ELISA technique. The highest spike represents with (X) is the highest level of rhoptry 5 protein the parasite expressed on the host (0.411), while lowest level of rhoptry 5 protein virulence (0.021) is represented by spike on level (Y).These highest spikes were above the optical density level of 0.261 from which microsporidia parasite is described to have become virulence.

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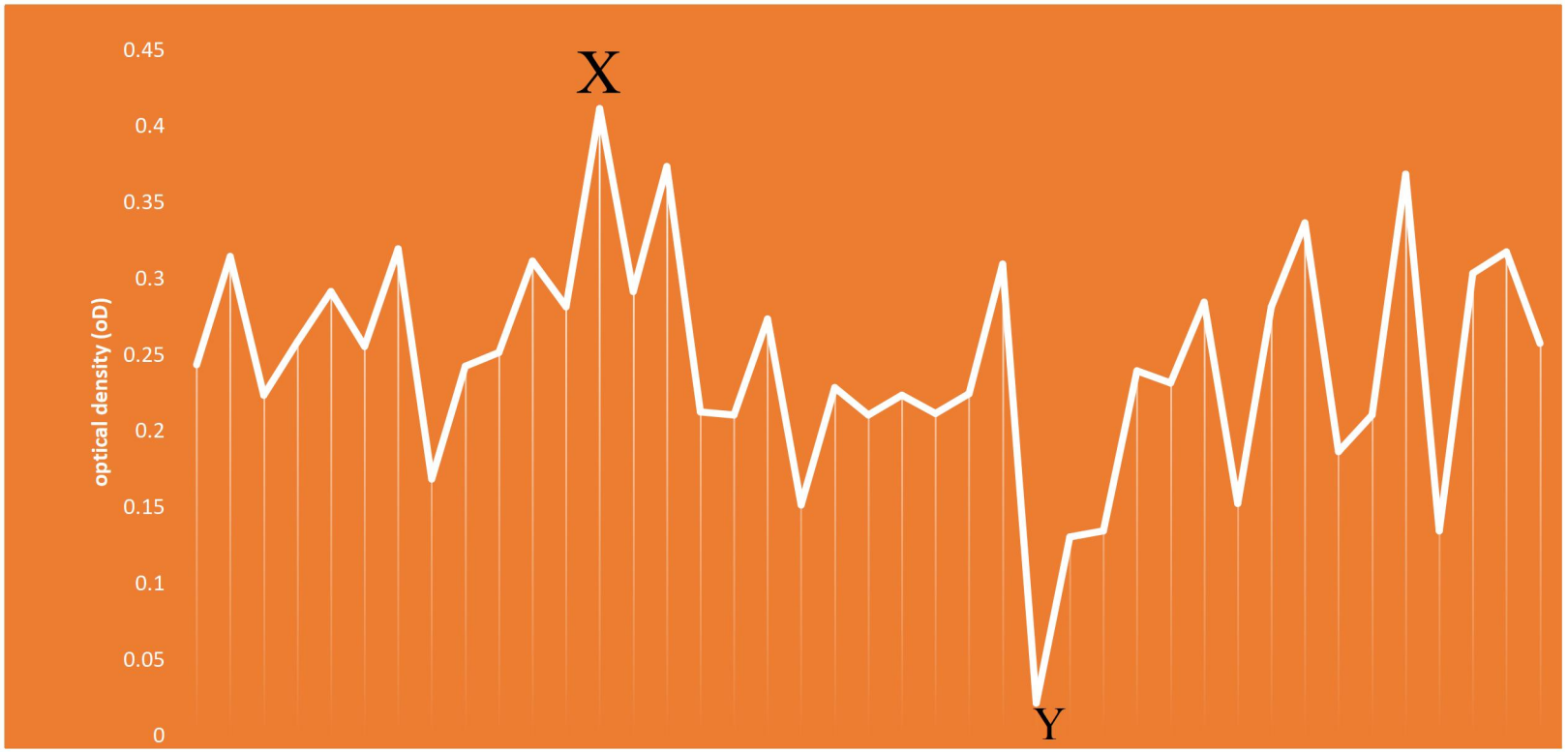

Rhoptry 5 (ROP5) protein kinase virulence level

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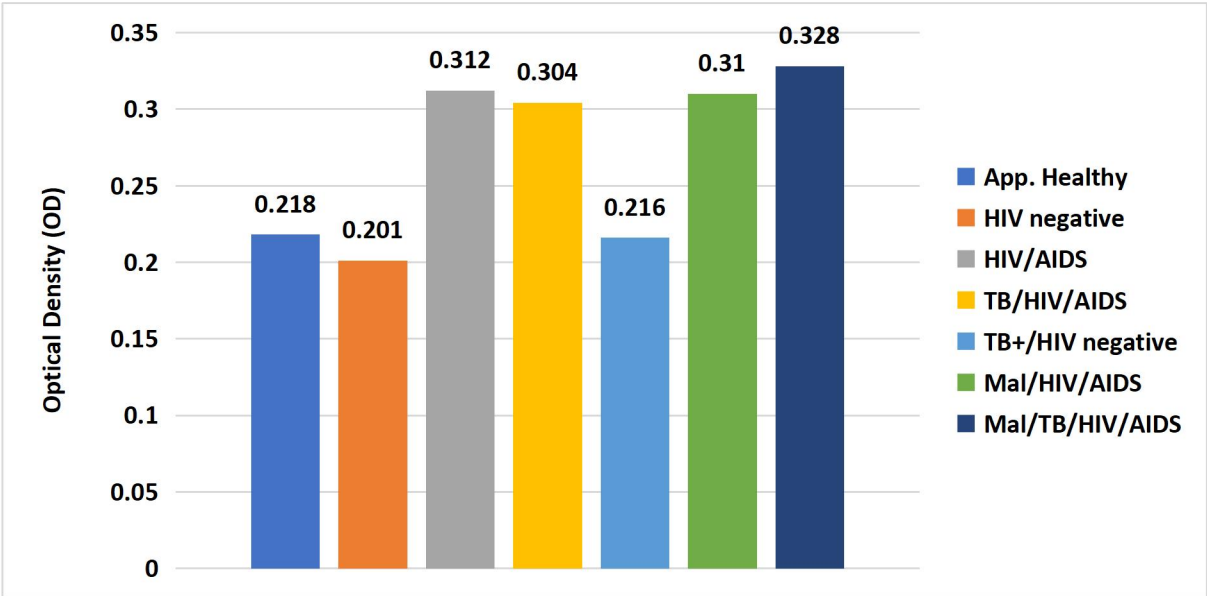
Rhoptry 5 (ROP5) protein virulence factor level in the different disease conditions.

The level of microsporidia rhoptry 5 protein virulence on the Apparenly healtyh and Immunocompromised patients that were positive for the infection indicates that the level of the rhoptry 5 protein virulence factor were above the Optical Density (OD) level in some positive patients from the study. Apparently Healthy participants, shows rhoptry 5 protein virulence of 0.218 OD, HIV negative had of ROP5 protein virulence of 0.201 OD. In Immunocompromised patients, highest protein virulence were recoded among Mal/TB/HIV/AIDS patients with 0.328 optical density, while the lowest were show among TB+/HIV negative patient 0.236 OD. HIV/AIDS had ROP5 protein virulence of 0.312 OD, while TB/HIV/AIDs patients had rhoptry 5 protein virulenc level of 0.304 OD and Mal/HIV/AIDA patients had ROP5 protein virulence level of 0.31 OD.



Rhoptry 5 (ROP5) protein virulence factor level in the different disease conditions.

Rhoptry 5 (ROP5) protein virulence factor level by age group

The level of rhoptry 5 proteins virulence of microsporidia were indicated in all ages, although some were not up to maximu of 0.261 optical density where microsporidia become obvious virulence to the host. Among the Apparently Healthy, ROP5 protein virulence level was highest within age 15 – 19 with optical density of 0.314, while the lowest of ROP5 virulence level of 0.224 OD was found in age groups 20 – 24years old. In HIV patient, the highest ROP5 virulence level was found at 50 years old and above 0.29 OD, while 0 – 14 years old had the least virulence level of 0.21 OD. Immunocompromised patients, the highest ROP5 virulence level in HIV/AIDS patients were from 50 years and above (0.319 OD) and least were from age 15 - 19 (0.242 OD). In TB/HIV/AIDS patients, the highest ROP5 virulence (0.411 OD) was found in 20 – 24 years old while the least ROP 5 protein virulence level (0.212 OD) was in 0 – 14 years old. The ROP5 was high (0.228 OD)

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in 15 – 19 years old among TB+/HIV negative patients while the least ROP5 (ROP5) protein virulence level (0.168 OD) was found in 0 – 14 years old. In Mal/HIV/AIDS highest ROP5 virulence were found in 0 -14 with 0.336 OD while the lowest was found in age 50 years and above 0.224. Highest ROP5 protein virulence was found in age 24 – 49 years old (0.368OD) in Mal/TB/HIV/AIDS patients while least was found in 50 years old and above 0.210 OD.

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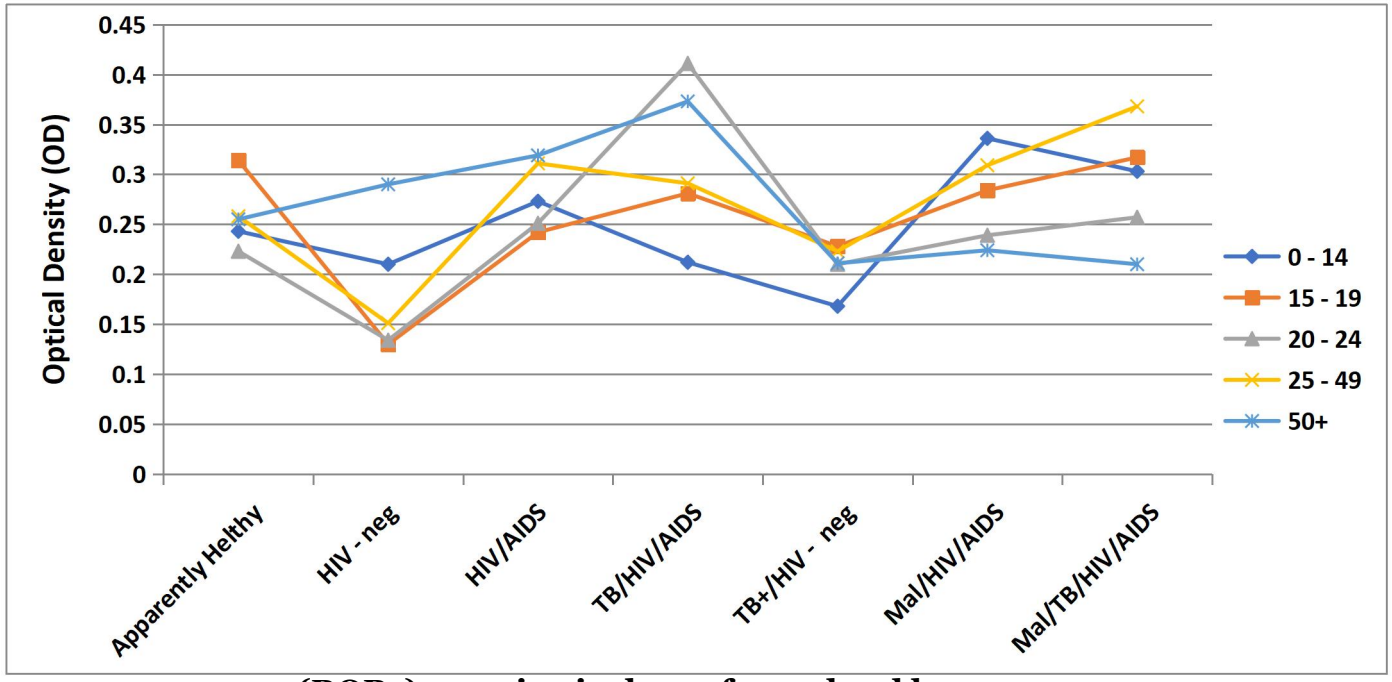

5 (ROP5) protein virulence factor level by age group

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DISCUSSION

In the past few years, rhoptry proteins specifically located in the rhoptry neck have been described and shown to take part in the formation of the so-called moving junction, a close apposition of the plasma membranes of both the parasite and its host cell, which allows the parasite to actively propel itself into the nascent parasitophorous vacuole and determine the level of severity of a parasite to the host. ROP kinase expansion is shared among closely related tissue cyst forming coccidians but they are now found broadly in the Apicomplexa (Yamamoto *et al*., 2011). In this reseach work, rhoptry 5 (ROP5) have been found in microsporidia to be the virulence factors used to attack their host with varing degree of optical density (OD) in different patients. According to manufacture manuer 0.261 is the minimum optical density that could not be virulence to the host. This is inline with work of (Peixoto *et al*., 2010) who started that half of the ROP kinases are predicted to be enzymatically active when orgnism with ROP attack their host, while the other half, like ROP5 lack an intact catalytic triad and are likely low catalytically competent. This could state the reason why some OD of some participant not above 0.261 OD where parasite become virulenceto their host. In contrary, in *T. gondii*, and Euccocidia protozoan parasites pseudokinase ROP5 forms complexes with the ROP17 and ROP18. ROP17 and ROP18 target distinct threonine residues in host immunity-related GTP their also act synergistically to control acute virulence of *T. gondii* in mice, ROP17 uniquely phosphorylates oligomeric IRGs, leading to their disassembly.

This research is in agreement with Behnke *et al*. (2015) that major virulence determinant in the mouse is ROP5, a polymorphic locus of tandem repeated genes that contributes to the acute virulence of type 1 strains, yet the corresponding cluster of ROP5 alleles in type II strains decreases virulence. Crystal structures are available for ROP2/ROP8 (Yamamoto *et al*., 2011), two pseudokinases that lack ATP binding, and also for ROP5 (Reese *et al*., 2011), which binds ATP in an unconventional manner, and it is unlikely to catalyze hydrolysis that was expresesd through optical density. Although, the virulence cut off mark by manufacturer's design was 0.261 Optical Density(OD) for microsporidia species, however some of optical density levels of infected patients were above the cut off mark OD. The lowest level of microsporidia rhoptry 5 protein kinase virulence was 0.130 OD while the highest virulence level recorded was 0.411 OD. According to (Niedelman *et al*., 2014) the major virulence factors ROP5 that have been identified in mice system target innate and adaptive immune responses that are important in control of infection is above 0.260 OD. Some of the participants whose OD recorded below the cut off marks of 0.261 have some form of virulence but the levels are not elagible for the parasite to express virulence as such, there is under expression of rhoptry 5 protein kinase virulence in such patient as described by Saeij *et al*. (2006).

A high screening assay for ROP 5 among the study population revealed that the virulence was highest (Mal/TB/HIV/AIDS patients and this could have resulted from heavy invasion of the host B – cells through N – terminal portion of virulence ROP5 as stated by (Behnke *et al*., 2015). The invasion could have been possible as a result of low immune status of the group. The lowest rhoptry 5 protein kinase virulence level was expressed in HIV negative. The low ROP 5 virulence level expressed in this group as a result of high immune status present in the group which could fight the parasite although the group

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still a carrier of the parasite in their blood system (Zhao *et al*., 2009) .

There has been a scanty reference report of microsporidia Rhoptry 5 protein level in relation to age groups. This study however, elucidate the virulence level of microsporidia rhoptry 5 protein virulence with age groups of individuals who participated. Among the Apparently Healthy, the virulence was highest in age group 15 - 19 years old (0.314OD) within this age their's evidence of high level of opital density, the least was found in age 0 – 14 years old (0.243), while HIV negative, the highest virulence level, 0. 290 OD, occur in 50 years old and above. HIV/AIDS patients had the highest virulence level (0.319 OD) in 50 years old and above. In TB/HIV/AIDS patients, the highest virulence level (0.411 OD) occurs in 20 – 24 years old while TB+/HIV negative patients had the highest virulence level (0.228 OD) in age group 15 - 19 years old. The mean value of the virulence level showed that the highest virulence level (0.284 OD) in the study population occur in the age limit 15 – 19 years old while the lowest virulence level in the population occur in 20 – 25 years old. This expline the reason most participants with OD more than minimu of 0.261 are weak and minurish in appearance.

Conclusion

The results provide new information on virulence factors ROP5 of microsporidia infection among apparently healthy subject and immunocompromised in Minna, The presence of varing degree of ROP5 virulence factore have shown in physical appearance of some patient whos OD is more tham 0.261 minimum level. On the different disease conditions, patient with complicated diseases had higher virulence ROP5 OD. More importantly, it has helped to determine the dynamic of microsporidia levels and virulence protein factors.

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