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A Study on the Ultra Structure of Actinomycosis in Women with IUDs

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Abstract: To investigate the structure of biofilm and the micro-structural characteristics of anaerobic Gram-positive Actinomyces by vaginal examination and microscopic analysis of the secretions from infected patients.The presence or absence of sulfur granules produced by these bacteria were analyzed using energy dispersive X-ray spectroscopy (INCA., Oxford Ins., UK) and clinical specimens from the patients infected with Actinomyces were collected for analysis via. Scanning Electron Microscopy (SEM). From high resolution Scanning Electron Microscopy (SEM), the surface of hypha-formed biofilmwas covered with a substance secreted by the bacteria and hexagonal sulfur granules were scattered aroundthe mycelium. The formation of the porous biofilm is believed to create the best conditions for bacterial growth and proliferation in the anaerobic state. Granules of 100-150 nm in size were observed and the components were measured by Energy Dispersive X-ray spectroscopy (EDX). Vaginal smear specimens of squamous intraepithelial cells were observed through optical, electronic and transmission electron microscopy and the bacteria were shown to produce an electrolyte salt. The most common of the bacteria is parasitic in the host and creates major growth of a biofilm that maintains a persistent infection. Ecosystems of the vagina are affected by a number of different types of microorganisms, pH and concentration of sugar. These ecosystems can also be affected by hormonal changes,medications, douches and frequency of sexual intercourse. Thus, it is considered that one should maintain personal hygiene such that the vaginal pH stays slightly acidic and one should use vaginal cleaning agents with special care, since, frequent use is considered to cause hypersensitivity reactions, total destruction of normal bacteria and a lowering of the vaginal pH.

Key words: SEM, TEM, EDX, Actinomycosis, sulfur granules, bacterial vaginosis

INTRODUCTION

Actinomyces belongs to Actinomycetales and grows in diverse directions. It is a non-acid fast anaerobic bacterium that shows PAS (Periodic Acid-Schiff)-and Gram-positive reactions (Simpson and Read, 2014). Moreover, this bacterium is opprotunistic, existing in the oral cavity and pharynx, ileocaecal and appendix as filaments with no formation of spores (Arend *et al*., 1998). Actinomyces are the most widely spread mycetoma among the existing bacteria and are classified according to the arrangement of growing hypha and spores and the structural features of the shape and surface of spores. The bacterium that causes Actinomycosis was identified in the late 19th century and actinomyces israelii has since, been shown to be the major pathogen that infects humans. *Actinomyces israelii* is the most common subtype causing disease of the Human body and coexists in an anaerobic state in the oral cavity, pharynx, tonsils, gastrointestinal tract and reproductive system (Fazili *et al*., 2012). Traditionally, actinomycosis appears as a result of an infection in the female reproductive system, ans has been shown to be derived from the gastrointestinal system. However, recent actinomycosis in the reproductive system is related to women who use an Intrauterine Device (IUD) (Kalaichelvan *et al*., 2006) with 20% of infected woman using such a device. Among infected patients, more than 25% show pelvic infection (Kayikcoglu *et al*., 2005). Actinomycosis is a chronic suppurative bacterial infection which was one of the most common diseases prior to the development of antibiotics, however, it rarely occurs now. It appares in the head and nech and spreads through the blood stream of tissue directly, causing swelling in the soft tissue of the head, face and neck and appears in the chest, lungs and chest wall. In smear samples of woman

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with an Intra Uterine Device (IUD), microorganisms similar to actinomyces are commonly found (Myer *et al*., 2005). For approximately 7% of the women withan Intra Uterine Device (IUD), *Actinomyces* spp. Appears in smear specimens (Norrington *et al*., 2008). In addition, women who wear the device for a long period of time show infection of the pelvis (Westhoff, 2007). Actinomyces infections in the female genital organs are increasing through contact with the perineum, uterine cervix, oral cavity or anus (Carrillo *et al*., 2010, Robert, 2009).

MATERIALS AND METHODS

Materials were selected targeting vaginal examination and the analysis of the vaginal secretions of inflammatory disease patients who had an Intra Uterine contraceptive Device (IUD) for more than 1 year and who presented with vaginitis and severe itching in H hospital. This study used clinical material fromvaginal examination and secretions that had been confirmed to be infected with Actinomycetes diagnosis by pathology specialists. Patient consent was obtained prior to the test. This study was executed following deliberation approval from the Public Institution of Bioethics Council appointed by the Ministry of Health and welfare (project management number: P01-201405-BS-03).

We collected samples from five female patients who had been diagnosed with intrauterine inflammationand actinomycos is in hospital H, located in Seoul. This study protocol was approved by the Ethics Committee of the Public Health Institutional Review Board, designated by the Ministry of Health and Welfare in Korea.

The samples of epithelial cells were collected from the endocervix and vagina by rotating the Rovers brush x in a clockwise direction 5 times and were subsequently placed in a liquid cytology solution toprevent cell degeneration. The Foreign materials (mucous and RBC) in the speculum were removed by cell prep® and a single layer of the samples was smeared on a glass slide.

Papanicolaou staining and light microscopy: To distinguish the Actinomycos is from the surrounding cells, the nuclei were stained with Harris hematoxylin solution and discolored using 0.5% hydrochloric acid (Young-Dong, Korea). The cytoplasm was also stained with Orange G-6 and EA-36 (Young-Dong, Korea). The samples were sealed using cover glass to prevent contamination and actinomycosis was observed under a light microscope (B×51, Olympus, Japan).

Scanning Electron Microscopy (SEM): Actinomycosis samples were pre-fixed and post-fixed and post-fixed in 2.5% paraformaldehyde-glutaraldehyde (4°C phosphate buffer, pH 7.4) and 1% OsO_4 (4°C, phosphate buffer), respectively, for 2 h. Fixed samples were washed with phosphate buffered solution (4°C, 0.4 M phosphate buffer, pH 7.4) several times and then dehydrated in increasing concentrations of ethanol (70, 80, 90, 95, 100%) which was then substituted with isoamyl acetate. The processed samples were dried in a critical point dryer (Hitachi SCP-II, Japan) and coated with gold at a thickness of 20 nm using an ion coater (JFC-1100, Japan). The samples were observed using a scanning electron microscope (JSM-840 A, Hitachi, Japan) at 20 kV.

Transmission Electron Microscopy (TEM): Actinomycosis samples were fixed in 2.5% paraformaldehyde-glutaraldehyde (4°C, phosphate buffer, pH 7.4) for 1 h, washed twice for 15 min with phosphate buffer (4°C, 0.4 M phosphate buffer, pH 7.4) and fixed again with 1% OsO_4 (4°C, phosphate buffer) for 1 h. The fixed samples were washed twice with the same buffer solution and then dehydrated in increasing concentrations of ethanol which was substituted with propylene oxide and then they were embedded in Epon-Araldite solution. Polymerization was performed at 60°C in a vacuum drying oven (Yamato, Japan) for 36 h. Semi-thin sections were cut from embedded samples using an ultrami crotome (Leica EM UC7, Germany) which were then stained with 1% toluidine blue (1% borax) on a hot plate (60°C) for 2 min. The stained sections were washed with distilled water and observe the microstructure of acinomycosis an ultra-thin section was cut and attached to a copper grid and then stained with uranyl acetate and lead citrate. This ultra-thin section was observed under a trasmission electron microscope (H-7500, Hitachi, Japan) at 100 kV.

Energy Dispersive Spectroscopy (EDX): Energy dispersive X-ray spectroscopy (INCA, Oxford Ins., UK) was used for the qualitative and quantitative analysis in the vaginal examination and clinical specimens diagnosed as actinomycosis at an acceleration voltage of 15 kV using the same method as with the Scanning Electron Microscope (SEM) observation.

RESULTS AND DISCUSSION

Optical microscopy of the specimens showed that Actinomyces colonies were stained dark blue byhemato xylin dye and many inflammatory cells were concentrated around them as like shown in Fig. 1.The edges of the hypha were observed as if they stick together and thin radiation filaments appeared projecting from the tangled clump in the center as like shown in Fig. 2. Scanning electron microscopy showed that Actinomyces were attached to the surface of vaginal speculum epithelial cells

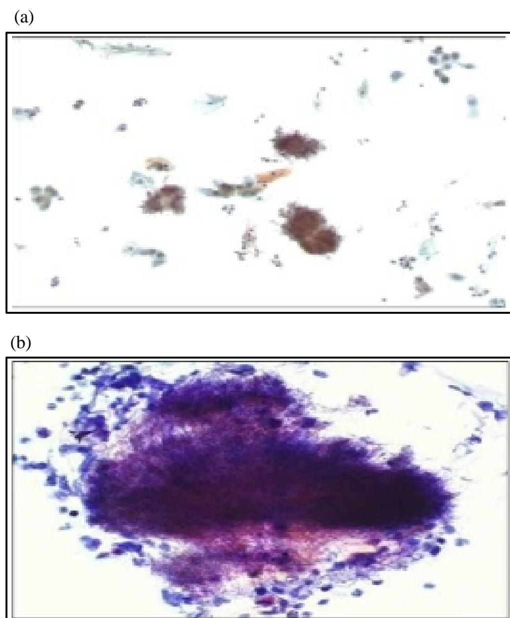

Fig. 1: Light micrograph of *Actinomyces* spp. showing the filamentous and branching bacterium. Pap. stain×100

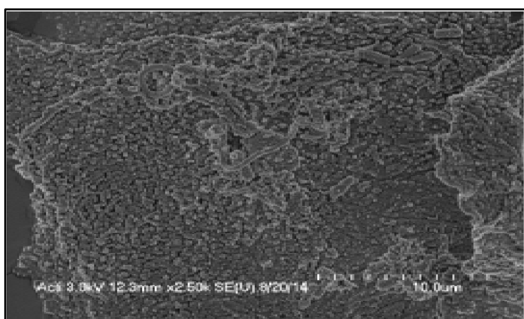

Fig. 2: Light micrograph of *Actinomyces* spp. showing the filametious clump. Pap. stain×400

with surrounding inflammatory cells with the mycelium extending in long branches. Thi smycelium formed the segment in an elongated state and branching was not observed as like shown in Fig. 3. High resolution Scanning Electron Microscopy (SEM) showed that the surface of the hypha formed abiofilm covered in a substance secreted by the bacteria and hexagonal sulfur granules were scattered aroundthe mycelium. These granular materials had a diameter of approximately 100-150 nm and were observed scattered around Actinomyces alone or together as like shown in Fig. 4.

The surfaces of epithelial cells to which Actinomyces were fixed were confirmed to be covered in biofilmand many pores existed on the surface where sulfur

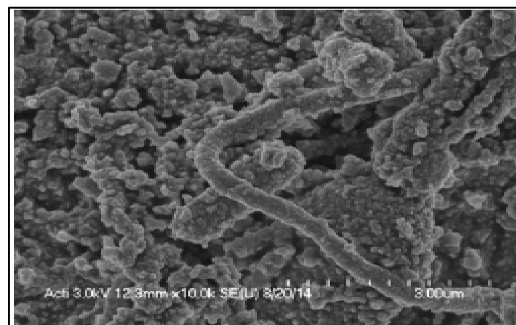

Fig. 3: SEM micrograph of Actinomyces biofilm on the epithelial cell surface

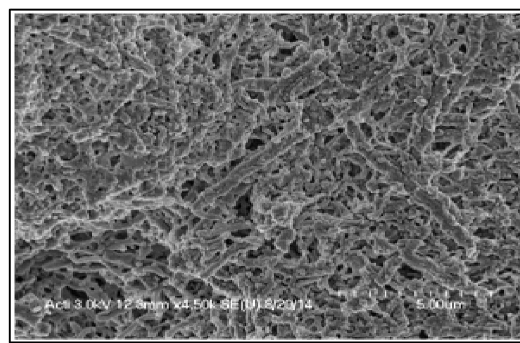

Fig. 4: SEM micrograph of Actinomyces biofilm showing substance production and sulfur granules

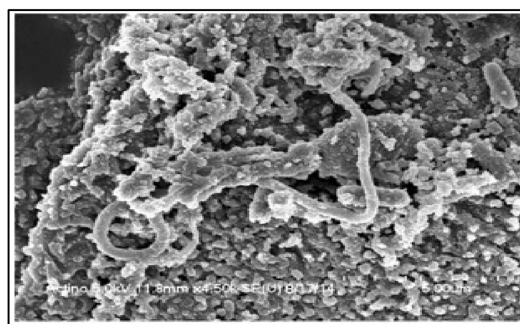

Fig. 5: Scanning electron micrograph of Actinomyces biofilm showing substance production and sulfur granules

granules were scattered as like shown in Fig. 5 and 6. Energy dispersive X-ray spectroscopy confirmed the existence of granules, composed of Carbon (C), Nitrogen (N), sodium (Na) and Sulfur (S) as like shown in Fig. 7. The sulfur (S) component and accountedfor 1.21% among the analyzed elements which was the highest with the exception of oxygen which can beexposed to the sample

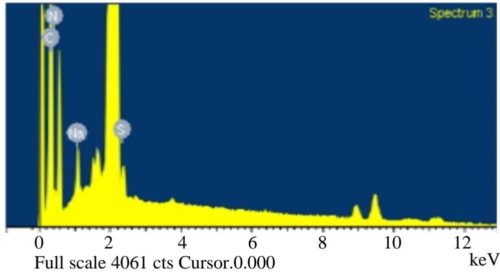

Fig. 6: Scanning electron micrograph of Actinomyces biofilm showing the hexagonal crystals observed inside and outside

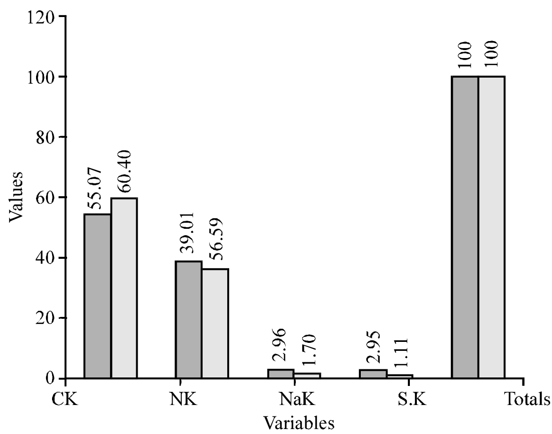

Fig. 7: Electron Dispersive X-ray spectroscopy (EDX) to analyze the Actinomyces biofilm composition

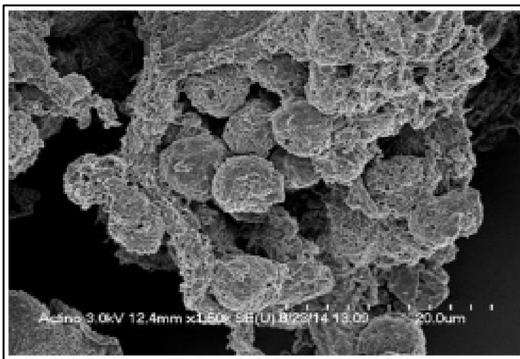

Fig. 8: Electron Dispersive X-ray spectrocopy (EDX) of the Actinomyces biofilm composition sshowing a sulfur element of 1.21%

during analysis, copper which is the supporting membrane component of thes pecimens and platinum which is the coating agent for Scanning Electron Microscopy (SEM) as Fig. 8. The Actinomyces that were entangled with the inflammatory cells were attached to the surface of these inflammatory cells, forming a biofilm and

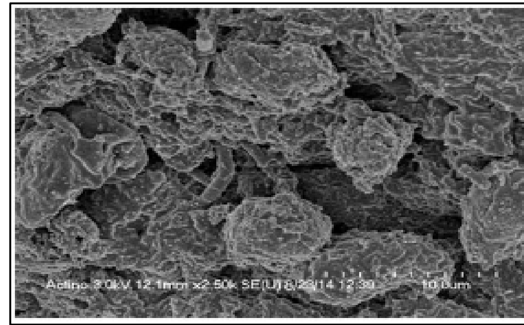

Fig. 9: Scanning electron micrograph of Actinomyces clumped with inflammatory leucocytes

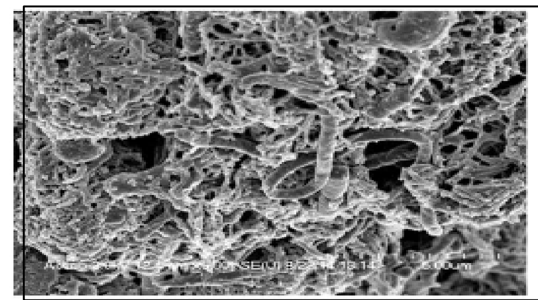

Fig. 10: Magnified scanning electron micrograph of Fig. 9. Actinomyces observed inside the inflammatory leucocytes and biofilm

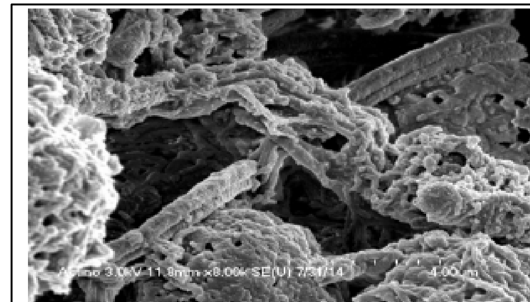

Fig. 11: Scanning electron micrograph of Actinomyces biofilm developedover a clump of inflammatory leucocytes

cells in bacillary form were observed around the inflammatory cells as like shown in Fig. 9 and 10 inflammatory cells that formed lumps with Actinomyces were observed to have decomposed or dissolved membranes a spherical shape and a diameter of 7-8 μm as like shown in Fig. 9. Actinomyces covered the surface of inflammatory cells, forming a biofilm for their division and growth were attached to inflammatory cells or connected inflammatory cells to the epithelial cells or

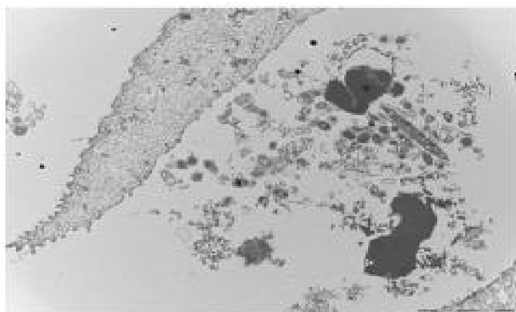

Fig. 12: Scanning electron micrograph of Actinomyces showing liner elongated mycelia

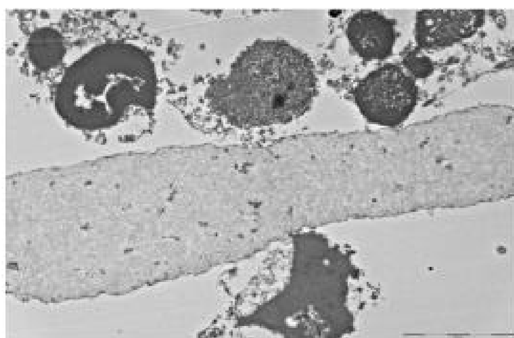

Fig. 13: Transmission electron micrograph of Actinomyces sample

bacteria to thein flammatory cells as like shown in Fig. 11 and 12. Transmission Electron Microscopy (TEM) showed that the mycelium and spores formed a colony and that epithelial cells were in close contact as like shown in Fig. 13. In addition, cells that formed lumps with Actinomyces had a concentrated or decomposed nucleus and cell organelle could not be observed as likeshown in Fig. 13. In particular, the cytoplasm of the epithelial cells was filled with cornification fibers and the nuclei of apoptotic inflammatory cells existed. The nuclei that were exposed because the cytoplasmwas completely dissolved or decomposed appeared to have a high electron density by the concentration of the nucleus or allow electron density due to fusion of nucleus as like shown in Fig. 14. Colonies of actinomyces under Transmission Electron Microscopy (TEM) occupied an area of approximately 30 μm^2 and mycelium and spores were distributed densely in a radial shape as like shown in Fig. 15. The mycelium and spores that were cut in various directions appeared to be different sizes and fibrous material in the space between the mycelium and the spore as like shown in Fig. 16. Spores in the breakup phase in high resolution Transmission Electron Microscopy (TEM) were confirmed

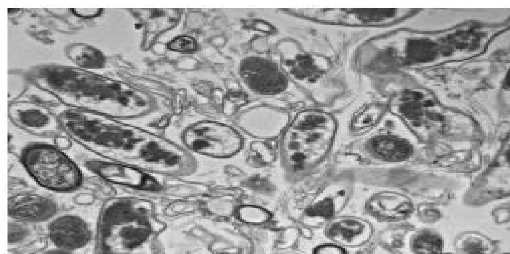

Fig. 14: Transmission electron micrograph of Actinomyces sample showing exfoliated epithelial cells andnaked nuclei

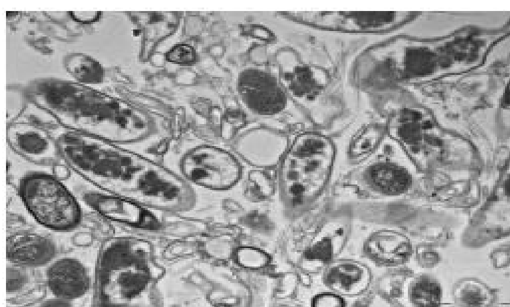

Fig. 15: Transmission electron micrograph of Actinomyces colony

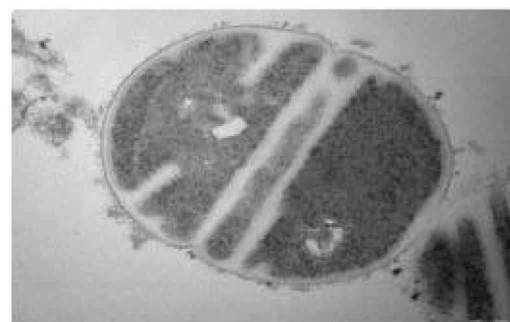

Fig. 16: Magnified transmission electron micrograph of Fig. 15 showing mycelia and spores

to form cell walls in the center and these cell walls appeared to have a low electron density with a thicknessof approximately 30 nm and the cell walls were observed clearly as like shown in Fig. 17. In addition, the cutting-edge in spores that had germinated formed thick cell walls as like shown in Fig. 18. Spores that stretched straight in transmission electron microscopy had a shorter diameter measuring approximately 0.3 μm as like shown in Fig. 19. In the mycelial cytoplasm, materials with a high electron density were scattered in the cytoplasm and the cell wall and nuclear membrane were also observed separately by a sharp boundary as

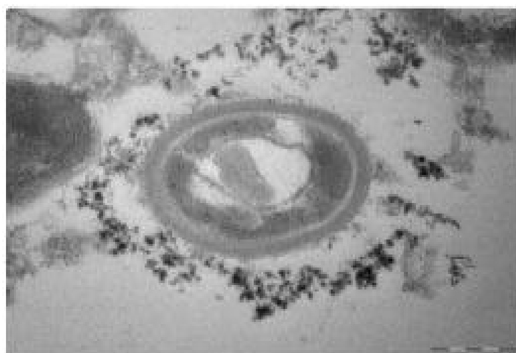

Fig. 17: High magnification transmission electron micrograph of Fig. 15 showing abudding spore

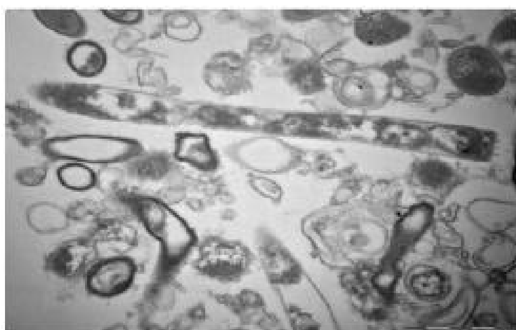

Fig. 18: High magnification transmission electron micrograph of Fig. 15 showing the spore

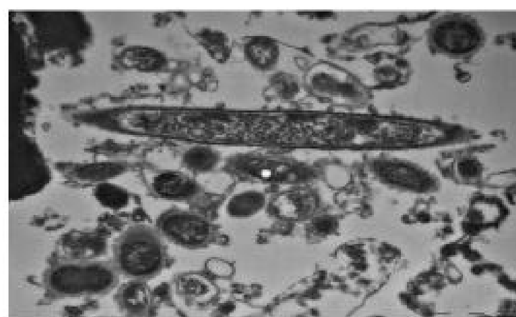

Fig. 19: Transmission electron micrograph of Actinomyces colony showing linear elongated mycelia

like shown in Fig. 19 and 20. On a section of Actinomyces specimen, the mycelium became large or showed various irregular shapes as like shown in Fig. 20. The spores of actinomyces in high magnification transmission electron microscopywere covered with biofilm as like shown in Fig. 21 and 22 and materials with a low electron density and amycelium thickness of approximately 40 nm surrounded the entire cell wall of the spores or were connected to adjacent mycelium or spores as like shown in Fig. 20 and 21.

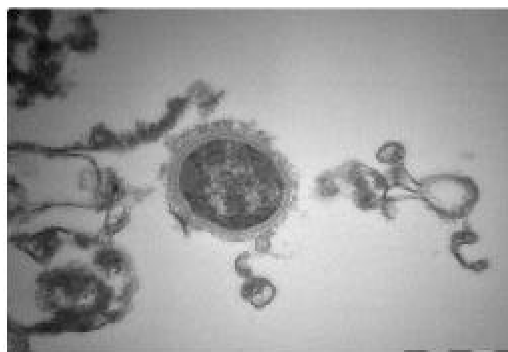

Fig. 20: Transmission electron micrograph of Actinomyces colony showing linear elongatedmycelia

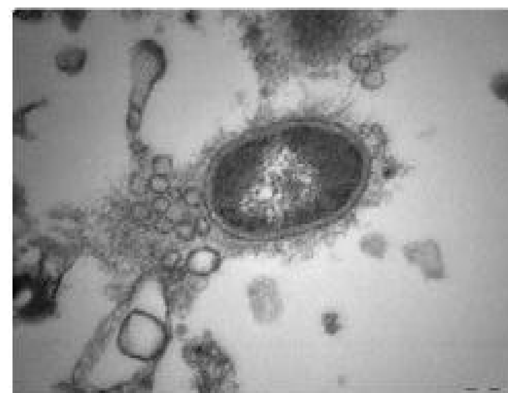

Fig. 21: High magnification transmission electron micrograph of Actinomyces covered with biofilm

CONCLUSION

This study aimed to investigate the structure of the biofilm and micro-structural characteristics of the bacteria that form as a result of actinomyces in the reproductive organs of women via. vaginal examination and visualization of the secretions of Actinomycosis using a light microscope. We also aimed to analyze the presence of avsence of sulfur granules produced by these bacteria using an Energy Dispersion Spectrum analyzer (EDX).

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