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IN VITRO IDENTIFICATION OF NAEGLERIA AND ACANTHAMOEBA ISOLATED FROM WATER AND SEWAGE

(With 10 Fig. & 3 Tables)

By

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(Received at 4/10/1993)

التصنيف المعملى للنيجليريا والاكانتاميبا المعزوله من مياه الشرب والمجارى

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فى هذه الدراسه تم عزل وتصنيف نوعين من الأميبا الممرضه وهما النيجليريا والاكانثاميبا وذلك من البيئه المائيه المصريه (مياه الشرب والمجارى) . وقد تم التصنيف على أساس الصفات المورفولوجيه - نوع وشكل النمو على بيئة SCGYEM وكذلك بيئة NM - بالاضافه إلى أختبار تحمل درجة الحراره. ولقد أسفرت الدراسه عن تصنيف أربعة أنواع من النيجليريا وكذلك ستة أنواع من الاكانتاميبا . ولقد وجدت بيئه NM ملائمة لاختبار ضراوة كل من النيجليريا والاكانتاميبا .

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SUMMARY

Potentially pathogenic free-living amoebae of the genera *Naegleria* and *Acanthamoeba* were isolated from the Egyptian aquatic environment (water and sewage). The isolated species of both genera were identified on the basis of morphological characteristics, type and form of growth in axenic SCGYEM medium and on monoxenic NM medium as well as the maximum temperature tolerance. The study revealed the identification of four species of *Naegleria* and six species of *Acanthamoeba*. SCGYEM medium was found to be more favourable for testing the pathogenicity of *Naegleria* only, while NM medium was favourable for testing both *Naegleria* and *Acanthamoeba* species.

INTRODUCTION

Infectious diseases continued to be the leading cause of morbidity and mortality in developing and industrial societies. Most of these infections were due to bacteria, fungi or viruses and least often due to protozoa and helminthes *(MARTINEZ, 1980)*.

Entamoeba histolytica had been recognized as the only being pathogenic to man and animals. This recognition effectively inhibited the consideration of the role of free-living amoebae as disease agents until the discovery of their cyto-pathogenicity in cell cultures by *JAHNES* et al. *(1957) and CULBERTSON* et al. *(1958)*. Since that time, small free-living amoebae had attracted the interest of both medical scientists and protozoologists.

Both pathogenic and non-pathogenic amoebae of genus *Naegleria* were found to contain a cytopathogenic material that act as an infectious agents *(DUNNEBACKE and DIXON, 1989)*. This phenomenon might explain the detection of specific antibodies against *Naegleria* in sera of healthy humanbeings, cattle and pigs by *CERVA, 1981 and 1989*.

Granulomatous amebic encephalitis and keratitis caused by *Acanthamoeba species* had occured world wide *(VISVESVARA and STEHR-GREEN, 1990)*. Also, they were found to be more resistant to free chlorine residuals than coliform bacteria. On the other hand, the survival of coliforms and other bacterial pathogens within Acanthamoebae had been confirmed by *KING* et al. *(1988)*. These findings indicated the tremendous public health implication due to the occurrence of Acanthamoebae in the aquatic environment.

So, the object of this work was to elucidate the presence or absence of potentially pathogenic strains of *Naegleria* and *Acanthamoeba* in the Egyptian aquatic environment with respect to their morphological and biological characteristics.

MATERIAL and METHODS

I. Collection of samples:

A total of 72,78,72 and 70 samples of tap water, well water, Nile water and sewage respectively were collected according to *APHA (1985)* from different localities in Giza province.

II. Laboratory isolation and identification:

for each sample, six plates were prepared, [three plates contained non-nutrient agar (NN) and the other three plates contained NN +1% sodium chloride (Nacl)]. Each plate was seeded with 0.1 ml. suspension of Escherichia coli as a food source for amebic growth.

For Nile water and sewage samples, each plate was inoculated with one ml. of the original samples. For tap and well water samples, each plate was inoculated with an inverted membrane (after filtration of 100 ml. original sample through the membrane).

Inoculated plates were divided into three groups. Each group contained one plate NN agar and one plate Nacl agar. The first group was incubated at 30°C, the second at 37°C and the third at 42°C for a period of 7 days with daily microscopic examination for the presence of any amebic growth.
plates showing amebic growth were subjected to the following :

1. *Direct examination of living amoebae:* Growing amoebae were picked up by a bacteriological loop and suspended in a drop of Page, s amoeba saline (*PAGE, 1967 a*) on a clean glass slide. Amoebae were examined microscopically for locomotion and general morphologey.

2. *Permanent stained preparations:* The previous directly examined slide (with amoebae) was fixed with several drops of Schaudinn' s fixative and allowed to stand for one minute. The slide was then transferred to a staining jar containing Schaudinn' s fixative and fixed for 30 minutes. Then, stained with H&E and/or Giemsa stain.

3. *Flagellation test:* The surface of agar plate was gently scraped by a bacteriological loop, then the loop contents were suspended in a test tube containing 1-2 ml. distilled water and incubated at the corresponding temperature of the plate from

which the scrapings were taken. Each 30 minutes, one drop of the tube contents was suspended in the concavity of a clean hanging drop slide and examined microscopically for flagella formation.

4. *Temperature tolerance test:* Amoebae from the original plates were subcltured on the same medium but incubated at an elevated temperature. The ager showing amebic growth was cut and inverted face to face on another fresh plate seeded with living suspension of Escherichia coli then, incubated at the desired temperature.

5. *Permanent stained cyst preparation:* Plates showing amebic growth kept at room temperature and after 5-6 days amoebae were found encysted on the plates. From these plates, slides were prepared, fixed and stained as in permanent preparations previously made for trophozoites.

6. *Cultivation on NM agar medium:* Temerature tolerant amoebae were subcltured on NM agar (*FULTON and DINGLE, 1967*) following the same method described previously in temperature tolerance test.

7. *Axenic cultivation in SCGYEM medium (CHANG, 1974):* Amoebae were harvested from ager plates, washed three times with page' s amoeba saline, resuspended in saline solution and concentrated by centrifugation. Then inoculated in the liquid axenic SCGYEM (serum-casein-glucose-yeast-extract medium) to which 200 units of penicillin and 200 ug streptomycin/ml. were added. Lastly, the inoculated medium was incubated at 37°C.

RESULTS

In this work, the obtained results showed conclusively that members of the genera *Naegleria* and *Acanthamoeba* existed in all types of the investigated water and sewage samples. The prevalence of the isolated strains of both genera are displayed in tables 1 & 2.

Genera of *Naegleria* and *Acanthamoeba* in this work were identified on the bases of the type of motility, ability to enflagellate and encyst, maximum temperature tolerance, the ability and form of growth in axenic medium (SCGYEM) and type of plaquing when grown on monoxenic medium (NM) beside other morphological characteristics.

Genus: *Naegleria:*

Morphologically, the isolated *Naegleria* species were very similar and differences were so minimal to depend upon in species identification. Differentiation of *Naegleria* from other amoebae was based mainly on their characteristic eruptive

movement of amoebic forms, their ability to enflagellate and encyst. Other features were used for species identification such as the rate of growth in axenic medium (SCGYEM), diameter of plaques appeared on monoxenic medium (NM) and their ability to grow at high temperature degrees.

The trophozoite of *Naegleria* was a vegitative form that had the ability to feed, divide, encyst and enflagellate. It was long slender or oval in shape measuring 12-35μ by 10-30μ (Fig. 1). It displayed a predominantly monopodal pattern of locomotion, usually progressed by a single lobose pseudopodium. This pseudopodium, usually erupted from the cell membrane in the form of hemispherical hyaline waves that determine the direction in which the amoebae would flow.

Flagellate form (Fig. 2) appeared when the amoebae were subjected to nutritional deprivation and the surrounding humidity increased (flagellation test). Firstly, the amoebae were swollen, become ronded and turned again to active lobate amoebae. Flagella began to appear and the body was elongated giving pear shaped actively swimming amoebae. Usually one pair of equal flagellae emerged from the pointed anterior end. The nucleus was located in the narrow anterior region having a distinct nuclear membrane and a centrally located prominant karyosome. Numerus cytoplasmic inclusions and vaculoes were present beyond the nucleus.

Flagellate stage was transient, non-feeding and unable to encyst. It can revert to amebic form within minutes or remained as a flagellate for several days. Reversion could be induced or enhanced by elevating the temperature to 37°C or more or mechanically by agitation for few minutes.

Encystment began when amoebae rounded up while their cytoplasmic vaculoes increased in number, decreased in size and became more active. Vac ucles disappeared gradually, cytoplasm reorganised and the karyosome became comparatively smaller. A thin rounded smooth cyst wall appeared in which small plugged pores (1-4) could be seen with difficulty.

A complete *Naegleria* cyst (Fig. 3) was spherical, measured 8-15U. No morphological differences were observed between different isolated species.

On applying culture techniques and heat tolerance test, examination of the isolated *Naegleria species* revealed the identification of four species namely, *N. jadini, N. gruberi, N. australiensis* and *N. lovaniensis.*

Genus: Acanthamoeba:

Members of genus *Acanthamoeba* included amoebae with only two forms of life, a trophozoite and cyst forms. Trophozoite

forms (Fig. 4) were easily recognized by the presence of broad hyaline lobopodia extending along sides, from which small filamentous hyaline projections (acanthopodia) extended. On the other hand, cyst form was characterized by the presence of double cyst wall (ectocyst and endocyst). There were plugged pores scattered on the surface of the cyst wall, the number, shape and arrangement of which differed from one species to another. Also, cysts had different shape which were species specific.

Cysts of different species of *Acanthamoeba* were morphologically distinct. So, it seemed applicable to distinguish them from each other by using the ordinary light microscope. Moreover, other features such as axenization in SCGYEM medium, monoxenic cultivation on NM medium and high temperature tolerance helped in declaring the pathogenicity of isolated species.

Examination of the isolated strains of Acanthamoeba revealed the identification of six species namely, *A. comandoni A. polyphaga, A. griffini, A. divionensis, A. lugdunensis and A. lenticulata.* The identified species were classified into three morphologically distinct groups according to *PUSSARD and PONS (1977.*

1. *Acanthamoeba species:* Group I: *A. comandoni* was the only isolated species belonging to this group. The cyst was 21-24 U in diameter and had 6-8 conical arms arranged in different planes giving the endocyst its star like appearance. Ectocyst was thin, transparent, nearly rounded and mostly surrounded the endocyst. Extremities of complex opercula were markedly bulged (Fig. 5).

It did not grow in axenic SCGYEM medium. When cultured on NM medium for 48 houres at 30°C, large plaques of more than 1.5 cm in diameter were obtained.

This species of *Acanthamoeba* was isolated from sewage (22.9%) and Nile water (12.50%) but could not be detected from both tap and well water samples (Table 2).

2. *Acanthamoeba species:* Group II: This group comprised most *Acanthamoeba* species revealed in the present study namely, *A. polyphaga, A. griffini, A. divionensis and A. lugdunensis.*

Acanthamoeba polyphaga had an average cyst diameter of 14 ± 1.2 U. Endocyst tended to be greatly irregular having small arms which were difficult to recognize. Ectocyst was moderately thin with a little dilatation, round or oval in shape and surrounded the whole endocyst. Complex opercula were small, much less apparent and did not protrude beyond the level of the endocyst (Fig. 6).

Acanthamoeba griffini had the average cyst diameter of 14 ± 0.75 U. Endocyst had different forms but it was usually globular or ovoid and had 4-6 massive and cylindrical arms which were enlarged at their extremities. Arms were characteristically disposed into two superimposed triangles. Ectocyst completely surrounded the endocyst, had thin transparent and irregular contour with granular cytoplasm at the periphery (Fig. 7).

Acanthamoeba divionensis had an average cyst diameter of 13.5 ± 0.35 U. Endocyst was globular and characteristically lemon shaped. Ectocyst was thin, transluscent and corregated. It covered the endocyst except at parts where pores appeared. The cyst had 5-6 massive and cylindrical arms located at parts uncovered with ectocyst. Cytoplasmic granules were markedly fine especially at the periphery of the cyst (Fig. 8).

These three species of *Acanthamoeba* did not grow in axenic SCGYEM medium while produced large plaques (more than 1.5 cm) when cultured on NM medium and failed to grow at 37°C. They were recovered from all types of investigated water and sewage (Table 2).

The cyst of *Acanthamoeba lugdunensis* had an average diameter of 14.2 ± 0.5 U. Endocyst was globular or ovoid in shape and had more or less an irregular contour. Endocyst formed 7 arms which did not protrude beyond the level of endocyst. Ectocyst was thin, fine, corregated and completely surrounded the endocyst. Cytpolasm was finely granulated at the periphery (Fig. 9).

A. lugdunensis did not grow in SCGYEM medium, while produced small plaques (less than 1.5 cm) when cultured on NM medium at 37°C for 48 hours. Also, it tolerated growth at 40°C. It was not recovered from both tap and well water while isolated from 2.8 and 2.9% of Nile water and sewage samples respectively (Table 2).

3. *Acanthamoeba, species.* Group III: Only *A. lenticulata* related to this group had been isolated in the present work. This species had an average cyst diameter of 12 ± 0.5 U. Ectocyst was thin with little undulation which surrounded the endocyst completely. Pores were difficult to be seen by ordinary methods. The cyst had fine peripheral cytoplasmic granules (Fig. 10).

A. lenticulata was grown with difficulty in SCGYEM medium giving a moderate type of growth. It produced small plaques (less than 1.5 cm) when grown on NM medium at 37°C for 48 hours. Also, it was found to tolerate growth at 40 and 42°C.

This species was only isolated from sewage samples in percentage of 2.9% but not recovered from different water samples (Table 2).

DISCUSSION

This work had been done in order to investigate the presence of potentially pathogenic free-living amoebae in Egyptian aquatic environment (water and sewage). To the best of our knowledge, previous studies on this subject in Egypt were recent and few. *MANDOUR et al. (1984)* investigated the pathogenicity of free living amoebae in Assiut province. *SADAKA et al. (1992)* studied the isolation and identification of free living amoebae from some water sources in Alexandria province.

Generally, the morphological characters of trophozoite, flagellate and cyst forms of *Naegleria* in the present study were in agreement with *MARCIANO-CABRAL (1988)*. They were too similar morphologically to be distinguished from each other at the level of ordinary light microscope. This finding was supported by *DE JONCKHEERE (1987 a)*. Some other biological features had been shared together in species differentiation such as the rate of growth in axenic SCGYEM medium, the shape of colonies on monoxenic NM medium and the high tamperature tolerance. These tests were considered sufficient for species identification of *Naegleria* by *RIVERA et al. (1981)*.

Our results explained that *Naegleria jadini* showed no evidence of growth when axenized in SCGYEM medium and could tolerate growth monoxenically only at 30 °C but not more. These findings were confirmed by *De JONCKHEERE (1987a)* and *MARCIANO-CABRAL (1988)*. When grown on NM medium, *Naegleria jadini* was found to form large plaques. In our opinion, the low maximum temperature tolerance (30 °C) and large plaques formed on NM medium, supported that *N. jadini* was a non-pathogemic species. This species was isolated from Nile water (11.1%), sewage (4.3%) well water (1.3%) but never reported from tap water (Table, 1).

The range limits of some physiological criteria of members of *Naegleria gruberi* were not strictly confined, as they could or could not grow under axenic condition. Also, they had a wide range of temperature tolerance up to 37 °C. These observations were coincided with *De JONCKHEERE (1987a)*. it was the most predominant species among all types of investigated water and sewage samples (Table, 1) *CURSONS et al. (1980)* related the predominancy of *N. gruberi* in tap water to it's resistance to chlorine more than other species of *Naegleria*.

Naegleria australiensis was isolated from all types of samples except tap water (Table, 1). This pathogenic species was found to give a moderate type of growth in SCGYEM medium, form small plaques when grown on NM medium and tolerate growth at 40 °C or even at 42 °C. It was recovered for the first time from the mud samples in Egypt during the collective study on the African continent by *DE JONCKHEERE and BAFORT (1989).*

The thermotolerant non-pathogenic *Naegleria lovaniensis* was isolated only from sewage samples (Table, 1). This species had characteristically grown at 45 °C but gave large plaques on NM medium and only a moderate growth in axenic SCGYEM medium. These criteria helped in the differentiation of *N. lovaniensis* from other thermotolerant pathogenic *Naegleria* which gave small plaques on NM medium. *I*t had been shown to act as a reservoir of infection with agents of legionnaires disease (*TYNDALL* and *DOMINGUE, 1982).*

Unlike *Naegleria,* species of *Acanthamoeba* could easily be distinguished from each other on the basis of cyst morphology. This was in accordance with the results obtained by *DE JONCKHEERE (1987 a).*

Acanthamoeba comandoni was the only species isolated in the present study belonging to *Acanthamoeba* group I. The morpological characteristics were distinct and agreed with *PAGE(1967b) and SAWYER (1989).*

Four species of isolated *Acanthamoeba (A. polyphaga A. griffini, A. divionensis* and *A. lugdunensis)* were found belonging to group II. Our results indicated that physiological characters of the first three species were nearly similar but they were morphologically distinct. They were recovered from all types of water and sewage samples and confirmed by *SCAGLIA* et al. *(1987)* and *ARIAS-FERNANDEZ* et al. *(1989).*

The pathogenicity of *Acanthamoeba lugdunensis* was evident in the present study through the formation of small plaques when grown on the NM medium. Besides, its ability to tolerate growth at 40 °C. Our finding was emphasized by *DAGGETT* et al . *(1982) and De JONCKHEERE, (1987a).*

The only isolated *Acanthamoeba* species belonging to group III was the pathogenic *A. lenticulata* . This species was found to give moderate growth in axenic SCGYEM medium, tolerate growth at 40 oreven 42 °C and gave small plaques on NM medium. It had been isolated from aquatic environment (*DAGGETT* et al., *1982*) and human nasal mucosa (*MICHEL* et al., *1982).*

In the present work, it was found that the rate of growth and differentiation of *Naegleria* and *Acanthamoeba* in SCGYEM reached 78.9 and 15.8% respectively (Table, 3). Therefore, SCGYEM proved to be suitable for differentiation of *Naegleria*

isolates only. These results were previously confirmed by DE *JONCKHEERE *1977) and (1980).*

On using the monoxenic NM medium, the differentiation between potentially pathogenic and non-pathogenic strains of *Naegleria* and *Acanthamoeba* reached 100 and 93.9% respecively (Table 3). Therefore, NM medium could be considered suitable for differentiation of both *Nagleria* and *Acanthamoeba* species agreeing in this result with *CURSONS* and *BROWN (1976)* who used the same medium for the same purpose.

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Table(1): Incidence of isolated species of Naegleria in different types of samples :

Species	Tap water		Well water		Nile water		Sewage		Total	
	No.	%	No.	%	No.	%	No.	%	No.	%
N. jadini	--	--	1	1.3	8	11.1	3	4.3	12	4.1
N. gruberi	7	9.7	20	25.6	33	45.8	30	42.9	90	30.8
N. australiensis	--	--	4	5.1	7	9.7	6	8.6	17	5.8
N. lovaniensis	--	--	--	--	--	--	2	2.9	2	0.7
Total	7	9.7	25	32.1	48	66.7	41	58.6	124	42.5

Table (2): Incidence of isolated species of Acanthamoeba in different types of samples.

species	Tap water		Well water		Nile water		Sewage		Total	
	No.	%	No.	%	No.	%	No.	%	No.	%
A. comandoni	--	--	--	--	9	12.5	16	22.9	25	8.6
A. polyphaga	1	1.4	3	3.9	5	6.9	3	4.3	12	4.1
A. griffini	4	5.6	6	7.7	9	12.5	25	35.7	44	15.1
A. divionensis	3	4.2	6	7.7	6	8.3	7	10.0	22	7.5
A. lugdunensis	--	--	--	--	2	2.8	2	2.9	4	1.4
A. lenticulata	--	--	--	--	--	--	2	2.9	2	0.7
Total	8	11.1	15	19.2	31	43.1	55	78.6	109	37.3

Table(3): Efficiency of both SCGYEM and NM media in differentiating Naegleria and Acanthamoeba species:

	Tap water		well water		Nile water		sewage		Total	
	No.	%	No.	%	No.	%	No.	%	NO.	%
Naegleria +ve plates	10		38		71		66		185	
Acanthamoeba +ve plates	14		22		46		83		165	
1. Axenizing in SCGYEM										
a. Naegleria growth	9	90	25	65.3	52	73.2	60	90.9	146	78.9
b. Acanthamoeba growth	1	7.1	4	18.2	5	10.9	16	19.3	26	15.8
2. Cultivation on NM										
a. Naegleria growth	10	100	38	100	71	100	66	100	185	100
b. Acanthamoeba growth	12	85.7	19	86.4	44	95.7	80	96.4	155	93.9

Number of examined samples: Tap water(72), well water(78), Nile water(72) and Sewage(70) [Total=292].

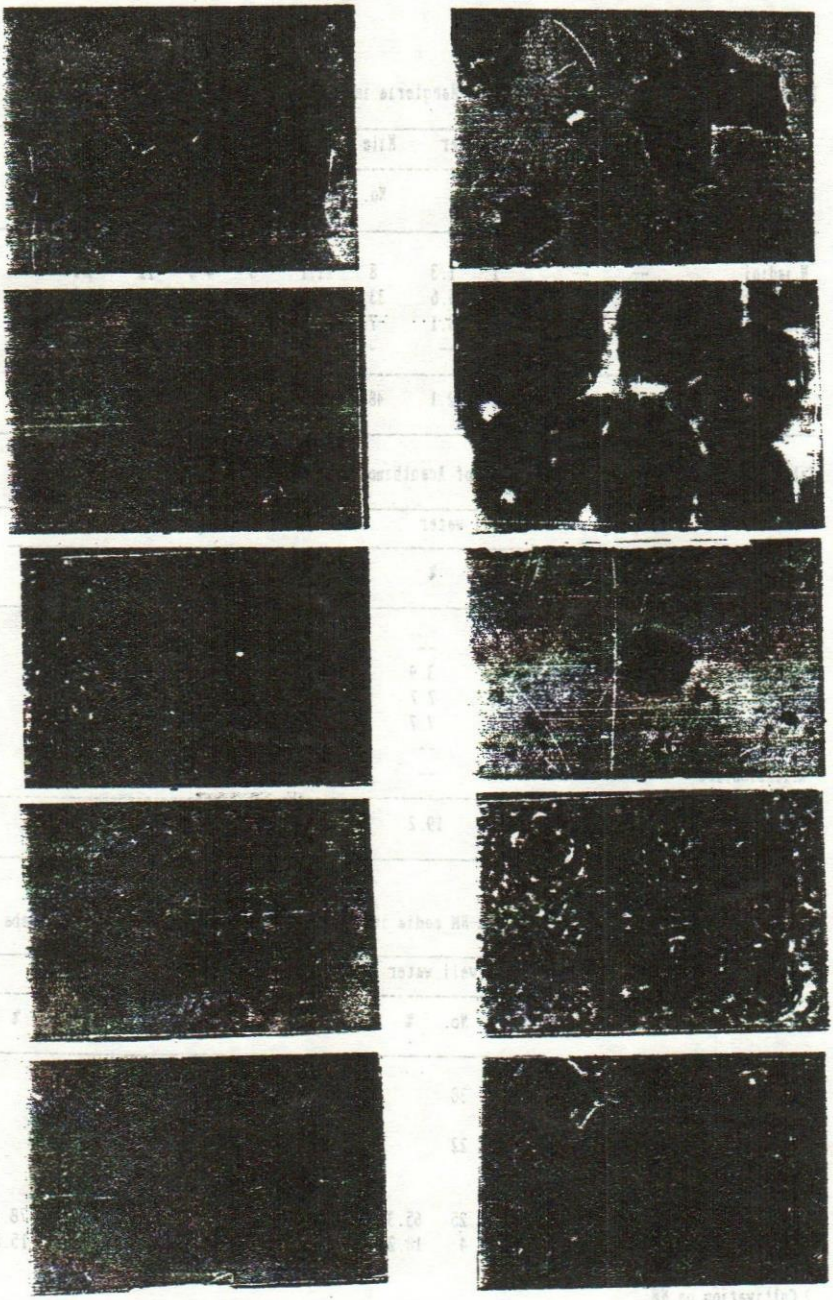

Fig.(1):Trophozoite of Naegleria species(X400).
Fig.(2) :Flagellate form of Naegleria species(X400).
Fig.(3):Cyst form of Naegleria species(X1000).
Fig.(4) :Trophozoite form of Acanthamoeba species(X400).
Fig.(5):Cyst form of Acanthamoeba comandoni (X400).
Fig.(6) :Cyst form of Acanthamoeba polyphaga (X400).
Fig.(7):Cyst form of Acanthamoeba griffini (X400).
Fig.(8) :Cyst form of Acanthamoeba divionensis (X400).
Fig.(9):Cyst form of Acanthamoeba lugdunensis (X400).
Fig.(10):Cyst form of Acanthamoeba lenticulata (X400).