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Microbiology and Pathology

Optimization of Citric Acid

Potent Phagocytic Microorganism

Highlights

Debre Markos Referral Hospital

Expression of Basal Cytokeratins

Discovering Thoughts, Inventing Future

VOLUME 17 ISSUE 2 VERSION 1.0



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Entamoeba Coli as a Potent Phagocytic Microorganism

By Mosab NM Hamad, Madiha E Elkhairi & Tarig M Elfaki

Elsheikh Abdallah Elbadri University

Abstract- Background: Entamoeba coli is intestinal protozoan amoeba which is regarded tell now as commensal amoeba although their adverse symptoms that they may cause in certain patients.

Objectives:

General Objectives: To know the phagocytic activity of Entamoeba coli against microorganisms.

Specific Objectives: To know the phagocytic activity of Entamoeba coli against microorganisms that inhabit the intestinal tract.

Methodology: The study based on data collected from previous studies.

Result: Entamoeba coli phagocytosed bacterial flora of the gut, fungi of Sphaerita species and even Giardia lamblia trophozoites.

Conclusion: Entamoeba coli is a potent phagocytic microorganism that engulf other microorganisms which may compete it in nutrients.

Keywords: entamoeba coli, phagocytosis, bacteria, parasite, fungi.

GJMR-C Classification: NLMC Code: QW 1



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the endoplasm. The nucleus is a ring like structure with thick nuclear membrane lined with irregularly distributed masses of chromatin and a large, irregular, eccentric karyosome.

Fine linin threads extend between nuclear membrane and karyosome. Trophozoite bears one to many pseudopodia which are short, blunt and granular. Movement is sluggish and usually not directional. The parasite feeds upon bacteria, vegetable cells and other faecal debris present in the large intestine. Dobell (1938) reported that it may ingest R.B.C., occasionally. The trophozoite reproduces by binary fission.

I. INTRODUCTION

Entamoeba coli are a protozoan endocommense, inhabiting the lumen of the large intestine of man. There is no reliable evidence that it produces disease in human beings but few workers have reported ingestion of red blood cells by the organism. E. coli was discovered in India by Lewis in 1870 however its detail description was given by Grassi (1879).

a) *Geographical Distribution*

It is cosmopolitan in distribution and has been stated to occur in about 50% of human population.

b) *Life Cycle*

Entamoeba coli are a monogenetic organism. Three distinct morphological forms exist during the life cycle-Trophozoite, Pre-cystic stage and Cystic stage.

Trophozoite of E. coli is about 20 to 30 in diameter with a range from 10 to 50. Trophozoite is unicellular. The cytoplasm is differentiated into outer narrow ectoplasm which is not so prominent and inner granular, vacuolated endoplasm containing bacteria and debris inside food vacuoles. A single nucleus lies inside

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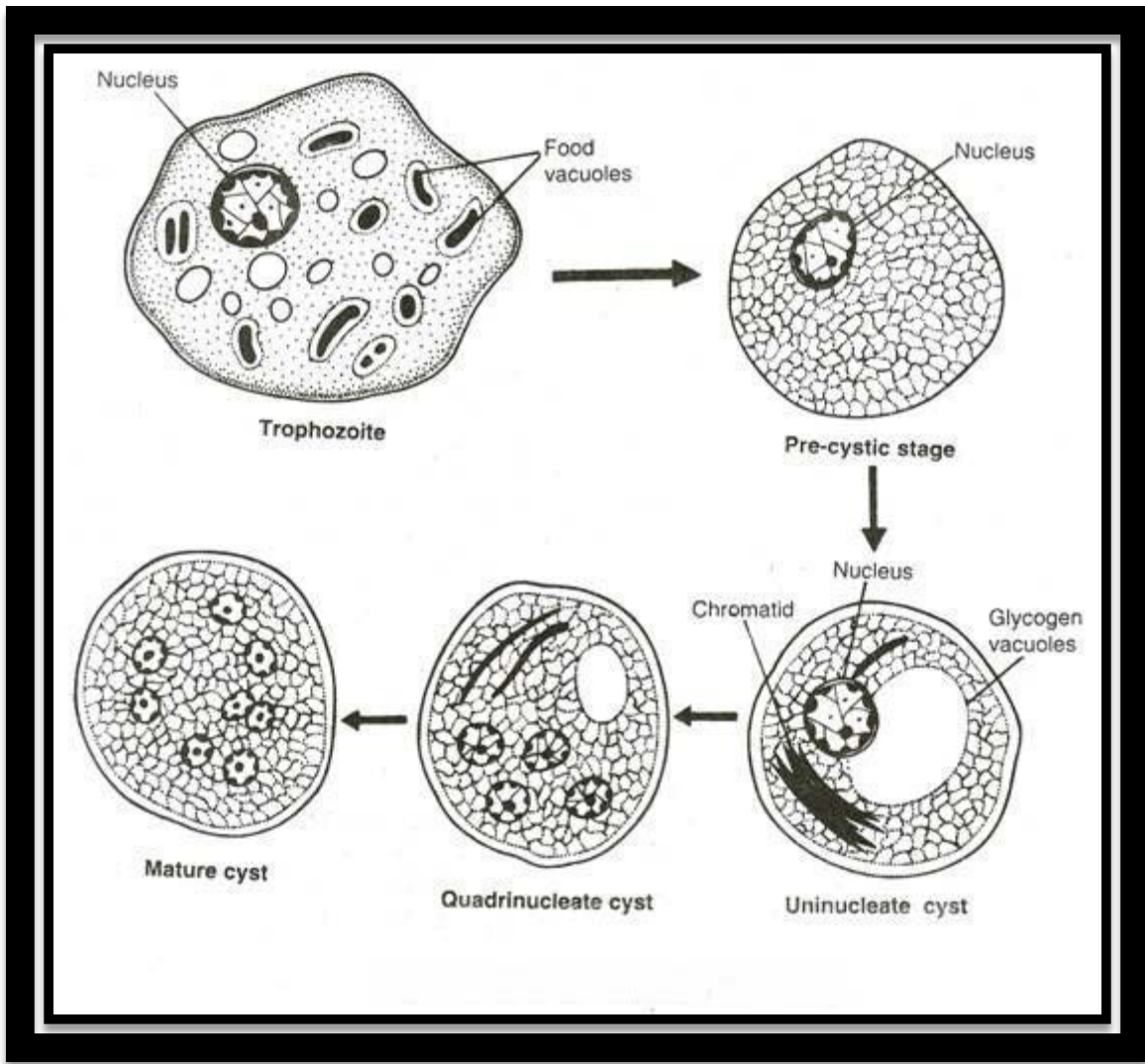


Figure 1: Stage of Life of Entamoeba Coil.

Trophozoite changes into spherical uninucleate precystic stage. The precystic stage size ranges from 15 to 45 μ in diameter. It is similar to trophozoite stage, except that it is non feeding stage and hence food inclusions are not found in the endoplasm. Precystic stage changes into cystic stage.

The cysts are spherical or aoid with size ranging from 10 to 33 μ m in diameter. The cyst wall is thick. Immature cyst may have one-two or four nuclei with eccentric karyosome .Occasionally, the cyst may bear 16 or even 32 nuclei. Glycoen vacuoles and chromatid bodies are seen in the endoplasm up to binucleate stage after that they are consumed. Matured cyst is the infective stage. Cyst formed in the large intestine is discharged out o the host's body through faeces. The cysts survive for 3-4 months outside the body of the host and are relatively more resistant to desiccation as compared to those of *E. histolytica*. The survive rate of the cyst is about 46 %.

c) *Mode of Infection*

Infection to the new host occurs by consuming contaminated food and drinks. The infective stage cysts are carried from faces to the food items through insects and rodents. In the small intestine of the new host excystation occurs during which a single multinucleate amoeba comes out through the cyst wall. Multinucleate amoeba divide into as many immature amoebas as there are nuclei in the cyst.

The young amoeba moves down to reach the caecum where they multiply in number and become trophozoites.

d) *Pathology*

E. coli lives inside the lumen of the large intestine in man. They never enter into the mucosa or sub-mucosa layers or other tissues of the intestine. There is no reliable evidence that it ever produces intestinal lesion, although it has been reported that *E. coli* occasionally ingest red blood cells.

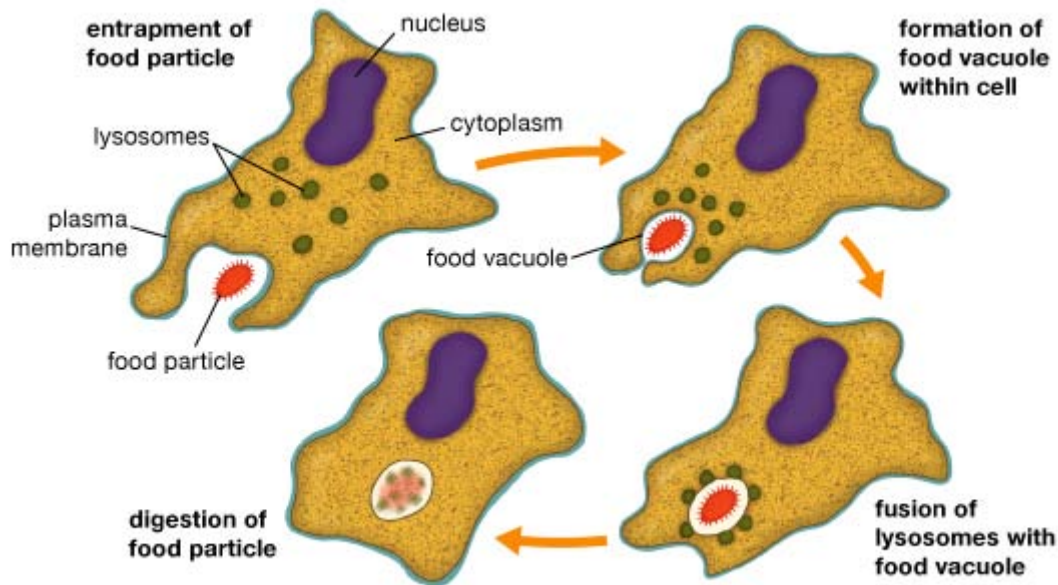
In this way it is believed to exist as non-pathogenic endo-commensal. However, Dey (1974) observed that a large population of *E. coli* inside the gut lumen may cause dyspepsia, hyperacidity, gastritis and indigestion.⁽¹⁾

Entamoeba coli feed on bacterial flora in GIT then it makes disturbance in bacterial flora functions.

Entamoeba coli has potent phagocytic activity through which it phagocytosed bacterial flora, fungi such as *Sphaerita* species and even other protozoan parasite such as *Giardia lamblia* trophozoite.⁽²⁾

e) *Phagocytosis*

Is a process by which certain living cells called phagocytes ingest or engulf other cells or particles. The phagocyte may be a free-living one-celled organism, such as an amoeba, or one of the body cells, such as a white blood cell. In some forms of animal life, such as amoebas and sponges, phagocytosis is a means of feeding. In higher animals phagocytosis is chiefly a defensive reaction against infection and invasion of the body by foreign substances (antigens).

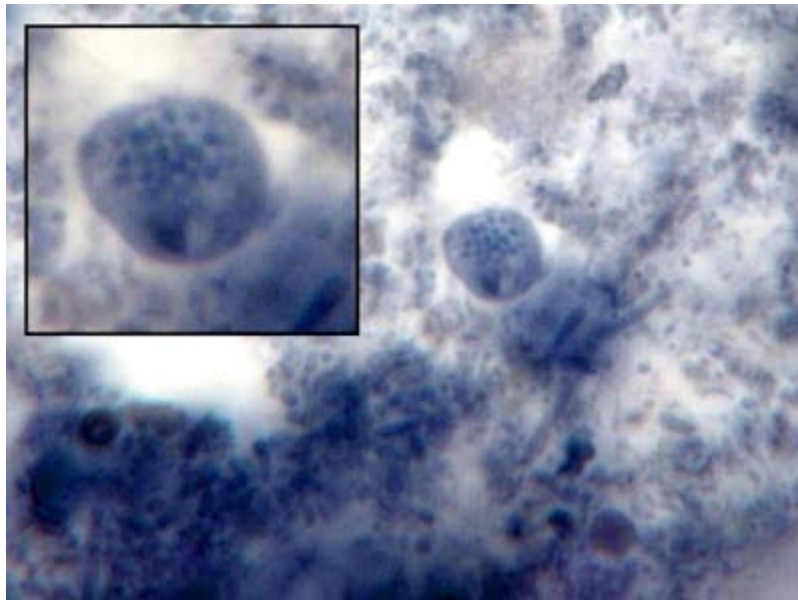


f) *Early Observation*

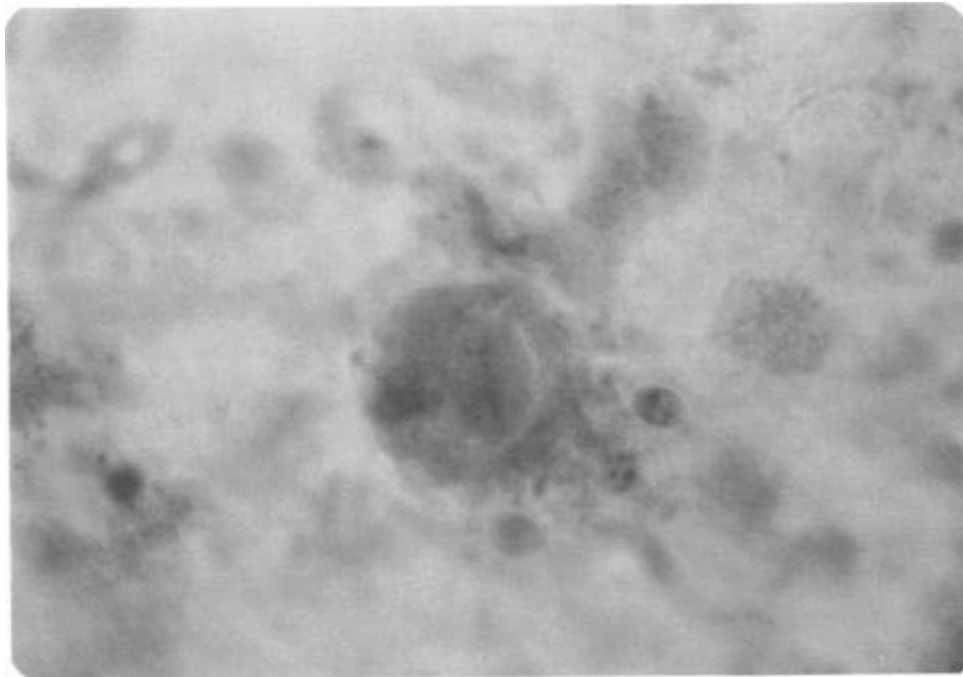
The presence of foreign particles within cells was first described in the 1860s by pathologist Kranid Slavjansky. In the 1880s Russian-born zoologist and microbiologist Élie Metchnikoff introduced the term *phagocyte* in reference to immune cells that engulf and destroy foreign bodies such as bacteria. Metchnikoff also recognized that phagocytes play a major role in the immune response, a discovery that earned him a share of the 1908 Nobel Prize for Physiology or Medicine.⁽³⁾

Some protozoan parasites can themselves be parasitized. A hyper-parasite! The genus *Sphaerita* is considered to be a lower fungus and some species are capable of invading the cytoplasm of some amoeboid parasites. Another parasite of parasites is *Nucleophaga* species which invades the nucleus. *Sphaerita*, (sometimes called *Polyphaga* spp.) appear as tightly packed clusters within the cytoplasm and measure approximately 0.5 μm to 1.0 μm .

The parasite show below is possibly an *Entamoeba coli*, however the nucleus is not visible as it is out of the plane of focus. *Sphaerita* appears as the small dots within the cytoplasm.⁽⁴⁾



Microscopic examination of a permanently stained fecal preparation revealed the unusual inclusion of a Giardia lamblia cyst within the cytoplasm of an Entamoeba coli trophozoite. ⁽⁵⁾



g) *Rationale*

Entamoeba coli till now regarded as nonpathogenic amoeba although their potent phagocytic activity that enable it to engulf other organisms whom compete it in nutrients and shelter.

h) *Objectives*

General Objectives: To know the phagocytic activity of Entamoeba coli against microorganisms.

Specific Objectives: To know the phagocytic activity of Entamoeba coli against microorganisms that inhabit the intestinal tract.

II. MATERIAL AND METHOD

Study Design: Observational study, data collected from previous studies.

III. RESULTS

From previous studies we knew that Entamoeba coli engulf certain microorganism and parasitized by others. And that showed potent phagocytic activity of Entamoeba coli.

IV. DISCUSSION

There is adequate agreement with others studies except that said Entamoeba coli is parasitized by Sphaerita species we suggested that Entamoeba coli phagocytosed that Fungal species as a part of their competition in nutrients and shelter.

V. CONCLUSION

Entamoeba coli had a potent phagocytic activity that enable it to engulf other competitive microorganisms.

VI. RECOMMENDATIONS

Another studies are required to know more about that potent phagocytic activity of Entamoeba coli.

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Prevalence of Multidrug Resistant Tuberculosis and its Associated Factors among Smear Positive TB Patients at Debre Markos Referral Hospital, Northwest Ethiopia

By Teabie Tsega, Dereje Gedle & Tebelay Dilnessa

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Abstract- Background: The emergence of MDR-TB is a threat for the populations of resource limited countries. In Ethiopia multidrug resistant tuberculosis is becoming a challenge, because of poor adherence to treatment, TB/HIV co-infection, and a few diagnostic and treatment facilities.

Objective: The main aim of this study was to assess prevalence and associated factor for multidrug resistant tuberculosis among smear positive TB patients.

Methods: A retrospective cross-sectional study was conducted among TB patients treated at DOT's clinic at Debre Markos Referral Hospital from September 1, 2015 to March 10, 2017. Data was entered and analyzed using SPSS version 20. Logistic regression was employed to assess associated factors with p -value <0.05 as significant.

Results: Of a total of 403 smear positive TB patients 248(61.2%), there was 48(11.9%) drug resistance TB cases. The prevalence of MDR-TB from both new and previously TB treated cases was found to be 1.5%. There was statically significant association between history of previous TB treatment and chance of developing MDR-TB. In this study previously treated patients have 34.26 times more likely to develop MDR-TB than treatment naïve patients [AOR= 34.26(95%CI: 4.89-24.11), $p=0.02$].

Conclusion: Previous history of TB treatment was found to be significantly associated with MDR-TB.

Keywords: MDR-TB, prevalence, HIV, tuberculosis.

GJMR-C Classification: NLMC Code: WA 400



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Conclusion: Previous history of TB treatment was found to be significantly associated with MDR-TB.

Keywords: MDR-TB, prevalence, HIV, tuberculosis.

I. BACKGROUND

Tuberculosis (TB) is an infectious bacterial disease caused by *Mycobacterium tuberculosis* that most commonly affects the lungs. Despite the recent progress of global control efforts, TB remains a major public health burden [1]. In 2014, there were 9.6x10⁶ cases and 1.5x10⁶ deaths of TB globally [2]. According to WHO Global TB report, Ethiopia is ranked as 15th among 27 high burden MDR-TB countries and 3rd in Africa. The estimated MDR rate was (0.9%–2.8%) for

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new cases and (5.6%–21%) for retreatment cases [3]. The history of TB treatment has observed sequential development of resistance to anti-TB drugs. Para-amino-salicylic acid and isoniazid were introduced to reduce the development of streptomycin resistance, which heralded the era of combination treatment for TB [4]. Treatment of MDR-TB using second line anti-TB drugs has more adverse events, since provided for an extended period of time (WHO recommendation at least 20 months) and is expensive [5].

Multidrug resistance TB (MDR-TB) is defined as tuberculosis caused by *Mycobacterium tuberculosis* resistant in vitro to the effects of Isoniazid and Rifampicin with or without resistance to any other drugs [6]. Primary resistance in TB refers to patients infected with *M. tuberculosis* that is resistant to anti-TB drugs from the outset, prior to anti-TB treatment. MDR-TB is essentially man made that emergence as result of poor TB control including poor supply of management and quality of anti-TB drugs, improper/inadequate treatment which is further fuelled by high prevalence of HIV [7].

MDR-TB is an emerging challenge for TB control programs globally. Emerging and spread of drug resistance TB has encountered as a great challenge in Africa region, Sub-Saharan Africa in particular. Information on the extent of MDR-TB from Africa region is very limited, probably due to poor laboratory facilities, poor surveillance mechanisms and reporting procedures, outdated databases and sub-optimal coverage of infrequent surveys. Sub-Saharan Africa stands the burden of both very high TB incidence and the highest HIV prevalence rates in the world, and represents 14% of the global burden of new MDR-TB cases [8]. Knowledge of the magnitude of MDR-TB is so crucial to allocate resources, and to address prevention and control measures [9]. Therefore, the aim of this study was to assess the prevalence of MDR-TB and associated risk factors at DMRH, Northwest Ethiopia.

II. MATERIALS AND METHODS

a) Study Area and Setting

A facility based cross sectional study was conducted among 403 TB patients who treated and

registered from September 1, 2015 to March 10, 2017 at Debre Markos Referral Hospital TB clinic. The hospital is a tertiary level hospital that provides health service for inhabitants in East Gojjam Zone and surrounding areas. It provides health service to more than 3.5 million populations in its catchments [10]. In the hospital, DOTS clinic was opened in 2012 under the National Tuberculosis and Leprosy Program of Ethiopia and it gives MDR-TB treatment services.

b) Sample Size and Sampling Technique

All smear positive TB patients registered and treated from September 1, 2015 to March 10, 2017 have been taken as a sample size. The study included 403 smear positive TB patients with full socio-demographic characteristics registered in the TB unit of Debre Markos Referral Hospital. The sample was taken orderly all patients' recorded and started TB treatment from September 1, 2015- March 10, 2017. Each individual TB patient's record had been selected from all tuberculosis patients' records using TB treatment card, TB register form, quarterly report on MDR-TB and case finding format at DMRH.

III. DATA COLLECTION AND ANALYSIS

Data was extracted by reviewing all the necessary registration formats from medical records and treatment charts in TB results at DMRH. The socio-demographic factors together with the clinical profile of the patient were extracted from medical records and treatment charts from the hospital TB database. The collected data were checked manually for the completeness and consistency and the data was cleaned, coded and analyzed using SPSS version 20. Logistic regression was used to determine the association between independent variables and the outcome variable. Odds ratio and 95% confidence intervals were calculated and the result was considered statistically significant at $p < 0.05$.

a) Ethical Consideration

The study was conducted after it is ethically reviewed by Department of Research and Ethical Review Committee of Debre Markos University. Then supporting letter was written to Debre Markos Referral Hospital. All information during data collection had been confidential; there were no any personal identification which was left on the check list.

b) Operational Definitions

- **MDR-TB:** Is defined as an MDR-TB suspect who is sputum culture positive and whose TB is due to Mycobacterium TB that are resistant in-vitro to at least isoniazid and rifampicin
- **New Cases of TB:** Is defined as a newly registered episode of TB or TB treatment for < 1 month
- **Re-treated cases of TB:** A previously treated case is defined as a newly registered episode of TB in a

patient with treatment history for TB for 1 or more month

- **Primary Resistance:** Patients with TB resistant to one or more anti-TB drugs, but who have never been previously treated for TB
- **Acquired Resistance:** Patients diagnosed with TB who start anti-TB treatment and subsequently acquire resistance to one or more of the drugs used during the treatment
- **Extensive Drug-resistance (XDR):** Resistance to any fluoroquinolone and at least one of the three injectable second line drugs (capreomycin, kanamycin, amikacin)
- **Mono Resistance:** Resistance to only one first line anti TB drug.

IV. RESULTS

a) The Socio-demographic characteristics of smear positive TB patients

A total of 403 smear positive TB patients were enrolled. Of these, 248(61.2%) were males and 155(38.8%) females (Table 1). The prevalence of drug resistance (both RR and MDR)-TB patients in this study was 48(11.9%) of which 29(60.4%) were males and 19(39.6%) females. Of all drug resistant TB cases only 6(12.5%) were MDR-TB and the remaining 42(87.5%) were mono-resistance (all rifampicin resistance). From all MDR-TB patients, 4(66.7%) were males and 2(33.3%) were females. Among 403 smear positive TB patients, 6(1.5%) were MDR-TB cases. Of all drug resistance TB cases, 43(89%) of them were rural dwellers and 5(11%) were urban dwellers. On the other hand all patients who were MDR-TB cases were rural dwellers (Table 3).

The highest number of drug resistance TB patients in this study were seen in 2016, 23(47.9%) followed by 22(45.8%) in 2015 and the least observed in 2017, 3(6.3%)(Table 3). Of all drug resistance TB patients across the study period, the age group 16-30 were the most affected which accounts 28(53.2%) followed by age group 31-45, 7(14.6%). The lowest number was observed in the age group 46-60, 3(6.3%). In contrast to this, the majority of MDR-TB patients, 4(66.7%) were in the age group of 0-15 and 16-30 each account 2 and the least were seen in the age group 31-45 and >60 which account 1(16.6%) in each group of the total 6 MDR-TB patients. The majority of smear positive TB patients, 355(88.08%) of them were susceptible for rifampicin AFB smear positive TB patients (pulmonary and extra-pulmonary) (Table 4).

b) Prevalence of smear positive TB among patients with age groups in different years

From a total of 25(6.2%) smear positive TB patients found in 0-15 age group, 13(52%) of them were males and the remained, 12(48%) were females. There was an increase amongst males in 2016, 6(46.2%) from the total 25 and amongst females in 2015, 6(46.2%). The

prevalence of smear positive TB patient with the age group of 0-15 was the same in 2015 and 2016, 8 in each year, but there was slightly increasing in 2017, 9 of the total 25 (Table 2).

The highest prevalence of smear positive TB patients in both male and female were observed in the age group 16-30 in 2015 and 2016. From the total number of 236(58.6%) smear positive TB patients in this age group, 142 (60.1%) males and 94(49.9%) females. Of these 72(30.5%) males and 48(20.3%) females were observed in 2015 followed by 52(22.0%) males and 33(24.3%) females in 2016. The least prevalence of smear positive TB patients were seen in 2017, 19(8.05%) males and 13(5.5%) females. In addition to this, the prevalence of male patients was relatively higher than that of females across the year 2015-2017 in this age group as indicated in Table 2.

In the age group 31-45, the percentage of smear positive TB patients increased from 2015-2016 and the highest was observed in 2016, 41(51.3%). The prevalence decreased from 41(51.3 %) in 2016 to 6(7.5%) in 2017. In addition to this, the percentages of males were relatively higher than that of females across the year from 2015 to 2017. The number of smear positive TB patients registered in this age group across the study period was 80(19.9%). Fifty one (63.8%) males and 29(36.2%) females were seen. The second highest prevalence 236(58.6%) was observed in age group 16-30 (Table 2).

The highest percentage of smear positive TB patients in the age group 46-60 was 23(50%) registered in 2015 followed by 17(36.9%) in 2016 and the lowest 6(13%) registered in 2017. The percentage of patients was decreasing from 2015, 23(50%) to 2016, 17(36.9%). The percentage of male patients was higher than that of female patients across the year from 2015-2016, but they are equal in 2017. The total percentage of smear positive TB patients among the age group >60 was 18(4.5%) registered from 2015-2017. Of these, 13(72.2%) were males and 5(27.8%) were females. The highest percentage was registered in 2015, 9(55.5%) and the lowest was 3(16.7%) observed in 2017. The percentage of male patients were relatively higher than that of females across the years from 2015-2017 (Table 2).

c) *Drug Resistance Pattern of TB and its Associated Factors*

Of all drug resistance TB patients, 39(81.3%) of them were pulmonary in site and from these 24(50%) of them were male and 15(31.3%) of them were females. The rest 9(19.7%) were extra pulmonary in site, of these 5(10.4%) were males and 4(8.3%) females. Of all MDR-TB patients, 4(66.7%) were belongs to pulmonary in origin and the remaining 2(33.3%) were extra-pulmonary TB (Figure 1). No statistically significant association was seen between site of TB infection and MDR-TB

[COR=0.8(95%CI 0.74-4.24, p=0.864)]. Forty three were rural dwellers and 5 were urban dwellers among 48 drug resistant TB cases. Therefore, significant association was observed between MDR-TB and residence [AOR=8.2 (95%CI 2.72-14.8), p=0.04] (Table 3).

Of all smear positive TB patients 292(71.2%), 163(56.7%) males and 129(43.3%) females have no previous history of TB treatment. The rest 111(29.8%), 76(65.5%) males and 35(34.5%) females were previously treated for TB. Among drug resistance TB patients the majority of them, 31(64.6%) were previously treated for TB, of these 18(58%) were males and 13(42%) females. Of 48 of drug resistance TB patients 17(45.4%) were new patients. Eleven (64.7%) were males and 6(33.4%) females. The highest number of previously treated drug resistance TB patients were seen in 2015, 17(35.4%) of these 9(52.9%) were males and 8(47.1%) females. All MDR-TB patients were previously treated for TB. Of these the majority, 4(66.7%) of them were under category 4 (failure of new regimen) and the remaining 1(16.6%) was under category 5(after failure of retreatment) and the rest 1(16.6%) was under category 2(relapse). There was statically significant association between history of previous TB treatment and the chance of developing MDR-TB [AOR= 34.26(95%CI: 4.89-24.11), p=002] (Table 3).

d) *Prevalence of Drug Resistance TB in HIV positive patients*

The prevalence of TB/HIV co-infection was 29(7.2%). Of these, 17(4.16%) were males and 12(2.9%) females. The majority of patients, 374(92.8%) were HIV negative. Age group 16-30 years, 19(4.67%) took the major share followed by 31-45 years, 9(2.19%) and the least affected age group was 46-60 and >60 each account 0% in retroviral infection among smear positive TB patients. Males were the most affected group in TB/HIV co-infection (Table 2).

The prevalence of drug resistance TB and HIV co-infection in this study was 12(24.9%). Among these 9(18.6%) were males and 3(6.3%) females. Across the study period males were predominate over females in drug resistance TB and HIV co-infection except in 2015, in this case both sexes were equal in number. On the contrary, none of MDR-TB patients were HIV positive. The highest number of drug resistance TB and HIV co-infection were seen in 2016, 8(12.5%) and the least (0%) were seen in 2017. There was no statistical significant association between drug resistant TB and HIV status [COR=22.5(95%CI=0.35-98.5, p=0.998)] (Table 3).

e) *Trends of MDR- TB across the study period*

From the total of 48 drug resistance TB patients, 42(87%) of them were RR (rifampicin resistance), of these 25(59.5%) were males and 17(40.5 %) females. Of all drug resistant, 6(12.5%) of them were MDR-TB patients, 4(66.7%) males and 2(33.3%) females. When observing the trends of MDR-TB across the study period

among smear positive TB patients, the number of MDR-TB patients were 3(0.74%), 2(0.49) and 1(0.24%) in 2015, 2016 and 2017 respectively. So the trends of MDR-TB was decreasing from 3(0.74%) in 2015 to 1(0.24%) in 2017 (Table 3).

V. DISCUSSION

The prevalence of MDR-TB from both new and previously TB treated cases in this study was found to be 1.5%.The finding in this study was lower than previous study in the same study area [11]. Multidrug resistance TB is estimated to be 3.7% of newly diagnosed patients with TB and 20% of previously treated patients around the world as shown by WHO 2012 report[8]. On the other hand, in the fourth WHO global report on anti-TB drug resistance in the world, data are reported from eight countries of the Region, and MDR-TB rate in this Region were 2% among new cases, 35.3% among previously treated cases and 5.4% from all or combined cases [6].In a previously study conducted from January 2011 to December 2013 stated that from a total of 2149 TB patients received in-patient treatment at St. Peter TB specialized referral hospital, 780(38%) patients were MDR-TB (culture positive) which is much higher than the result of this study [12].

A study finding in Northeastern China showed the prevalence of MDR-TB of 8.7% [13]. Similar study findings in New Delhi, India, shows from sputum positive pulmonary TB clients enrolled, the prevalence of MDR-TB among newly diagnosed pulmonary TB patients was 1.1% [14]. Another study in India on the pulmonary TB drug resistant shows 8% MDR-TB [15]. In contrast, all the above mentioned results were higher than the result found in this study and all MDR-TB patients were previously treated for tuberculosis, but there were MDR-TB cases in treatment naïve patients.

A research conducted in Dessie town, among 434 TB cases of TB treatment, 9(2.1%) were found to be MDR-TB cases which is a bit higher than this study finding [16]. On the other hand, a study which was conducted in Addis Ababa, at St .TB Specialized hospital from January 2011 to December 2013, a total of 2149 TB patients were received in-patient TB treatment in this hospital, of which 780(38%) patients were MDR-TB [12]. This higher prevalence of MDR-TB might be due to most of the patients were referral cases. Other studies conducted in the same study area at Debre Markos referral hospital showed, the prevalence of MDR-TB was 2.3% and 3% which is higher than the current study [11, 17]. But most MDR-TB cases were observed in males, 16-30 age groups and rural dwellers in agreement to the current study.

The result is slightly higher than the result of this study. But gender distributions of MDR-TB patients were almost similar to the previous studies. Regarding the

trends of MDR-TB in the study area, previous study shows an increasing trend of MDR-TB patients across the study period which was in contrast to this study. The trend of MDR-TB was 0%, 0.3%, 0.6%, 0.5% and 0.9% for 2011, 2012, 2013, 2014, and 2015 respectively. The trend of MDR was increasing in the study area from 0.0% in 2011 to 0.9% in 2015[18-22]. The decrease in the prevalence of MDR TB in the current study area may be due to better information of the community about the cause, transmission, prevention and treatment of tuberculosis. And commitment of the health professionals to strictly follow TB patients during the intensive and continuation phase of TB treatment. The new cases MDR-TB prevalence of this study was found to be null (0%) from all smear positive TB cases, which was extremely lower compared to other studies mentioned above. The possible reason for this low figure finding could it be low MDR-TB detection status of the hospital. On the other hand, the higher prevalence of MDR-TB in previously TB treated patients may be due to a poor adherence of patients to anti TB drugs by different reason.

There was no statistically significant association between age groups and MDR-TB occurrence from this study, which is similar in a study finding in Dessie administration[16]. In contrast to this, age was considered a risk factor for MDR TB as it was explained in a previous study, Ethiopia[23].Age group at 25-44 years in Bangladesh was a risks factor of MDR-TB. Sex was not significantly associated with MDR-TB according to this study finding. Similarly, there was no statistically significant association between sex and MDR-TB occurrence in a study conducted Dessie city administration in Ethiopia[16]. In contrast to this a nationwide survey conducted in China showed that, female gender were a risk factors for MDR-TB [13]. But a study in Nigeria showed gender was not significantly associated with MDR-TB [24]. Another study finding in Thailand also showed male gender as risk factors for MDR-TB [25]. In Ethiopia, male gender was a risk factor for MDR-TB in previous study [26] which is in contrast to current study.

Regarding treatment status of MDR-TB patients, all 6(100%) of them were previously treated for TB. And there was statically significant association between history of previous TB treatment and the chance of developing MDR-TB. In this study previously treated for TB patients have 34.26 times more risk to develop MDR-TB than treatment naïve patients. This result is similar to the report in a previously conducted study in Dessie City administration, Ethiopia stated that prevalence rate of MDR-TB from new TB cases, retreated cases and combined of all were found to be 0.3/100, 21.6/100 and 2.1/100respectively from all forms of TB cases. The prevalence rate of acquired MDR-TB was similar to the combined prevalence rate above since all MDR-TB

cases were acquired whereas, primary MDR-TB rate was null [16].

The prevalence of MDR-TB in previous study was 2.3% of which 0.2% new cases and 2.1% previously treated cases. Drug resistance was strongly associated with previous treatment [27, 28]. This is comparable with the result of this study. In this study, there was no statically significant association between occurrences of MDR-TB and HIV status of patients. This result is similar to the fining in Kenya, Malawi, Tanzania, Cote d'Ivoire and France [29-32].The Global Project of MDR-TB, which has been gathering data since 1994 from 7 countries, none of which have a high prevalence of HIV infection and there was no association between HIV infection and MDR-TB in 5 of these countries, where as a significant association was observed between MDR-TB and HIV infection in 2 countries Latvia and Ukraine [33,34].HIV was a risk factor for TB/MDR-TB accordingly to, WHO report at California; US during 2011which shows HIV contribute 4.5% MDR-TB cases [8].

With respect to the contribution of site of TB to multidrug resistant in the current showed that from all MDR-TB cases 4(4/6) of them were from pulmonary TB type and the rest 2 (2/6) were extra-pulmonary. A comparable result was noted in another study in Ethiopia which showed pulmonary TB type was a risk factor for MDR-TB [26].In contrast to the result of this study,a study in southern Ethiopia showed that HIV have statistically significant association for both acquired MDR-TB and primary MDR-TB [27].

VI. CONCLUSION

Prevalence of MDR-TB for both new and retreated TB cases from all smears positive TB patients at DMRH were found to be 1.5%. Previous history of TB treatment was found to be significantly associated with MDR-TB. In this study, age, sex and HIV status were not associated with MDR-TB. Counseling related to anti-TB drugs adherence during intensive and continuation phase of TB treatment is mandatory to decrease MDR-TB.Further prospective study is necessary to have more information about MDR-TB in the country in general and in study area in particular.

a) *Limitation of the Study*

The data was collected by using secondary data source from already recorded documents, so there were some difficulties in getting all the necessary information regarding the study across the study period. Even thought, the hospital started to give MDR TB diagnosis and treatment in February, 2014, the study included data only for 3 years (2015-2017) due to time constraint.

b) *Abbreviations*

DMRH: Debre Markos Referral Hospital, DMU: Debre Markos University, DOTs: Directly Observed

Therapy's, DST: Drug Susceptibility Test, EMB: Ethambutol, EFMOH: Ethiopian Federal Ministry of Health, HIV: Human Immunodeficiency Virus, INH: Isoniazid, MDR-TB: Multi Drug Resistant Tuberculosis, OR: Oddis Ratio, PAS: Para Amino Salicylic Acid, RMP: Rifampicin, STM: Streptomycin, TB: Tuberculosis

c) *Ethical approval and consent to participate*

The study was approved by research and ethical review committee of Debre Markos University. All information during data collection was confidential; there was not any personal identification which was left on the check list.

d) *Consent for Publication*

Not Applicable.

e) *Availability of data and materials*

All data generated and analyzed during this study were included in the manuscript.

f) *Competing Interests*

Authors declare that they have no competing interests.

g) *Funding*

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Table 1: The socio-demographic characteristics of smear positive TB patients by age group, sex and retrovirus infection status at DMRH, Northwest Ethiopia, March 2017, (N=403)

| Age group | Sex | | | HIV Status | | | | | |
|-----------|-----|-----|-------|------------|----|-------|----------|-----|-------|
| | M | F | Total | Positive | | | Negative | | |
| | | | | M | F | Total | M | F | Total |
| 0-15 | 13 | 11 | 24 | 1 | 0 | 1 | 12 | 11 | 23 |
| 16-30 | 142 | 92 | 234 | 12 | 7 | 19 | 130 | 85 | 215 |
| 31-45 | 51 | 29 | 80 | 4 | 5 | 9 | 47 | 24 | 71 |
| 46-60 | 29 | 17 | 46 | 0 | 0 | 0 | 29 | 17 | 46 |
| >60 | 13 | 6 | 19 | 0 | 0 | 0 | 13 | 6 | 19 |
| Total | 248 | 155 | 403 | 17 | 12 | 29 | 231 | 143 | 374 |

Table 2: The prevalence of smear positive TB patients among age groups and HIV status in different years at DMRH, Northwest Ethiopia

| Year | Age group | Sex | | | | Total | HIV Status | | | | | | | | |
|------|-----------|-----|------|----|-------|-------|------------|------|---|------|-------|----------|----|-------|--|
| | | M | % | F | % | | Positive | | | | | Negative | | | |
| | | | | | | | M | % | F | % | Total | M | F | Total | |
| 2015 | 0-15 | 2 | 25 | 6 | 75 | 8 | 1 | 100 | 0 | 0 | 1 | 1 | 6 | 7 | |
| | 16-30 | 71 | 59.7 | 48 | 40.3 | 119 | 6 | 60 | 4 | 40 | 10 | 65 | 44 | 109 | |
| | 31-45 | 19 | 57.6 | 14 | 42.4 | 33 | 5 | 71.4 | 2 | 29.6 | 7 | 14 | 12 | 26 | |
| | 46-60 | 14 | 60.9 | 9 | 39.1 | 23 | 1 | 100 | 0 | 0 | 1 | 13 | 9 | 22 | |
| | >60 | 8 | 80 | 2 | 20 | 10 | 0 | 0 | 0 | 0 | 0 | 8 | 2 | 10 | |
| | Total | 114 | 59.1 | 79 | 40.9 | 193 | 13 | 68.4 | 6 | 31.6 | 19 | 101 | 73 | 174 | |
| 2016 | 0-15 | 6 | 75 | 2 | 25 | 8 | 0 | 0 | 0 | 0 | 0 | 6 | 2 | 8 | |
| | 16-30 | 52 | 61.2 | 33 | 38.86 | 85 | 2 | 66.7 | 1 | 33.3 | 3 | 50 | 32 | 82 | |
| | 31-45 | 28 | 62.3 | 13 | 37.7 | 41 | 2 | 40 | 3 | 60 | 5 | 26 | 10 | 36 | |
| | 46-60 | 12 | 70.6 | 5 | 29.4 | 17 | 0 | 0 | 0 | 0 | 0 | 12 | 5 | 17 | |
| | >60 | 3 | 60 | 2 | 40 | 5 | 0 | 0 | 0 | 0 | 0 | 3 | 2 | 5 | |
| | Total | 101 | 64.7 | 55 | 34.3 | 156 | 4 | 50 | 4 | 50 | 8 | 97 | 51 | 148 | |
| 2017 | 0-15 | 5 | 55.6 | 4 | 44.4s | 9 | 0 | 0 | 0 | 0 | 0 | 5 | 4 | 9 | |
| | 16-30 | 19 | 59.4 | 13 | 40.6 | 32 | 2 | 100 | 0 | 0 | 2 | 15 | 11 | 26 | |
| | 31-45 | 4 | 66.7 | 2 | 33.3 | 6 | 0 | 0 | 0 | 0 | 0 | 2 | 1 | 3 | |
| | 46-60 | 3 | 50 | 3 | 50 | 6 | 0 | 0 | 0 | 0 | 0 | 3 | 3 | 6 | |
| | >60 | 2 | 75 | 1 | 25 | 3 | 0 | 0 | 0 | 0 | 0 | 2 | 1 | 3 | |
| | Total | 33 | 58.9 | 23 | 41.1 | 56 | 2 | 100 | 0 | 0 | 2 | 27 | 20 | 47 | |

Table 3: Resistance pattern and treatment categories of MDR-TB patients at DMRH Northwest Ethiopia from September 2015-March 10, 2017

| Year | Treatment Status | | | | | | | | Resistance Type | | | | | | | | | |
|-------|------------------|------|---|------|--------------------|------|----|------|-----------------|------|-------|------|----|-------|------|---|------|---|
| | New | | | | Previously Treated | | | | RR | | | MDR | | | | | | |
| | M | % | F | % | M | % | F | % | M | F | Total | M | F | Total | | | | |
| 2015 | 4 | 80 | 1 | 20 | 9 | 52.9 | 8 | 47.1 | 11 | 57.9 | 8 | 42.1 | 19 | 2 | 66.7 | 1 | 33.3 | 3 |
| 2016 | 6 | 60 | 4 | 40 | 8 | 61.5 | 5 | 39.5 | 13 | 61.9 | 8 | 38 | 21 | 1 | 50 | 1 | 50 | 2 |
| 2017 | 1 | 50 | 1 | 50 | 1 | 100 | 0 | 0 | 1 | 2.1 | 1 | 2.1 | 2 | 1 | 100 | 0 | 0 | 1 |
| Total | 11 | 64.7 | 6 | 35.3 | 18 | 58 | 13 | 42 | 25 | 59.5 | 17 | 40.5 | 42 | 4 | 66.7 | 2 | 33.3 | 6 |

RR- Rifampicin resistance, MDR- Multidrug resistance

Table 4: Analysis of socio-demographic and clinical factors for MDR-TB patients at DMRH, Northwest, Ethiopia, March 2017

| Variables | | Resistant Pattern | | COR (95% CI) | P-value | AOR (95% CI) | P-value |
|----------------------------------|----------|-------------------|-----|------------------|---------|--------------------|---------|
| | | R | S | | | | |
| Sex | M | 29 | 219 | 1.6(0.06-9.11) | 0.574 | - | |
| | F | 19 | 136 | 1 | | | |
| Age | 0-15 | 6 | 28 | 1 | | | |
| | 16-30 | 28 | 206 | 0.73(0.02-0.86) | 0.340 | - | |
| | 31-45 | 7 | 73 | 0.33(0.21-5.3) | 0.997 | - | |
| | 46-60 | 3 | 43 | 0.86(0.81-7.2) | 0.997 | - | |
| | >60 | 4 | 15 | 0.41(0.24-10.29) | 0.768 | - | |
| Previous history of TB treatment | Yes | 31 | 80 | 31.0(9.78-74.44) | 0.001 | 34.26(4.89-24.11) | 0.002 |
| | No | 17 | 275 | 1 | | 1 | |
| HIV status | Positive | 12 | 17 | 22.5(0.35-98.5) | 0.998 | - | |
| | Negative | 36 | 338 | 1 | | | |
| Residence | Rural | 43 | 200 | 5.4(1.64-10.63) | 0.025 | 8.2(2.72-14.8) | 0.04 |
| | Urban | 5 | 108 | 1 | | 1 | |
| Site of TB | PTB | 39 | 238 | 0.8(0.74-4.24) | 0.864 | - | |
| | EPTB | 9 | 70 | 1 | | | |

R=Resistant, S= Sensitive, PTB=Pulmonary Tuberculosis, EPTB=Extra-pulmonary Tuberculosis

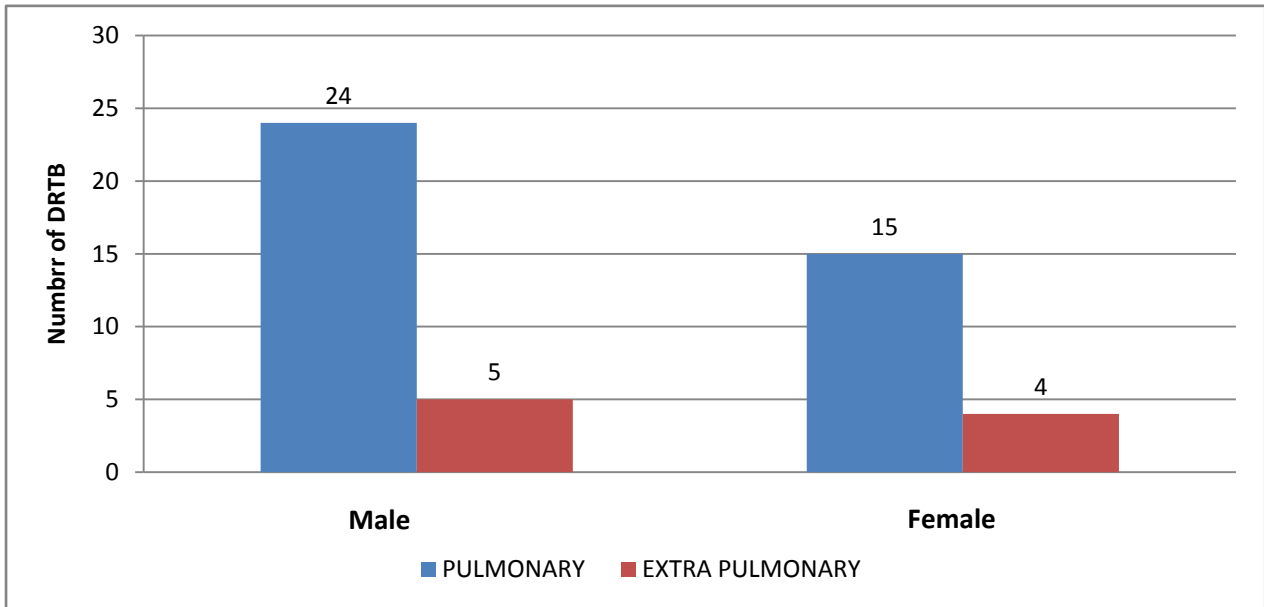


Figure 1: Site of drug resistance TB among patients at Debre Markos Referral Hospital, Northwest Ethiopia, March 2017



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The Expression of Basal Cytokeratins in Breast Cancers

By Dr. Vidhya Lakshmi S & Dr. Seyed Rabia

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Abstract- Introduction: Treatment for breast cancer is based on the expression of the immunomarkers such as ER, PR and HER2/neu. Cases which are negative to all the three immunomarkers, are called Triple Negative Breast Cancers (TNBC) and they have a poor prognosis. Recent studies have shown that some of the TNBCs express cytokeratins CK 5/6 (subcategorizing them as basal-like breast cancers) and these respond well to anthracycline-based chemotherapy.

Aim and Objectives: To study the expression of basal cytokeratins CK 5/6 in breast carcinomas reported in our centre and to correlate with histological type, grade, size, clinical features and ER, PR and HER2/neu status.

Methods: Tissues of 44 cases of breast carcinoma diagnosed between June 2009 and May 2014 were retrieved. Immunohistochemical staining for CK 5/6 was done and it was correlated with parameters such as histopathological type, grade, size, invasion and ER, PR and HER2/neu status.

Keywords: triple negative breast cancers, cytokeratin 5/6, basal-like breast carcinoma.

GJMR-C Classification: NLMC Code: QU 475



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The Expression of Basal Cytokeratins in Breast Cancers

Dr. Vidhya Lakshmi S^α & Dr. Seyed Rabia^σ

Abstract- Introduction: Treatment for breast cancer is based on the expression of the immunomarkers such as ER, PR and HER2/neu. Cases which are negative to all the three immunomarkers, are called Triple Negative Breast Cancers (TNBC) and they have a poor prognosis. Recent studies have shown that some of the TNBCs express cytokeratins CK 5/6 (subcategorizing them as basal-like breast cancers) and these respond well to anthracycline-based chemotherapy.

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Results: Eight of the breast carcinomas (18%) were categorized as Triple Negative Breast Cancers (TNBC) as they were negative for ER, PR and HER2/neu. Four of the TNBCs (50%), were positive for CK 5/6. Significant statistical correlation was observed between the size of the tumour and positive CK 5/6 expression. All CK 5/6 positive cases were of high grade.

Conclusion: The routine use of CK 5/6 is recommended in all cases of TNBCs, as 50% of them are positive for these markers. Patients in this subcategory could benefit from anthracycline-based chemotherapy.

Keywords: triple negative breast cancers, cytokeratin 5/6, basal-like breast carcinoma.

I. INTRODUCTION

Breast cancers are a diverse group of diseases that vary remarkably in terms of clinical presentation, histology, behavior and genetic characteristics [1]. There has been a steady increase in the incidence of breast cancers worldwide and especially in the developing countries, mainly attributable to globalization causing adaptation of western lifestyle and improved access to diagnostic modalities. As per the International agency for research on cancer, the number of new cases of female breast cancers in India in the year 2012 was 144,937. [2] The mortality rate in the Indian cohort was 50% compared to that in USA, where only one woman out 5-6 patients die of breast cancer.

Breast cancers that express Estrogen and Progesterone receptors can be treated by hormonal

manipulation [3]. Targeted therapy towards HER2 neu has great success and Trastuzumab has been introduced as an adjuvant drug in those showing over expression of Her 2 neu [4]. A subset of breast cancers have been found to show no expression of any of the above mentioned markers. These have been labelled as Triple Negative Breast Cancers (TNBCs). Though hormonal manipulation is of no use in this subset, they have found to show expression of other markers such as basal Cytokeratins and EGFR. They have greater sensitivity to anthracycline based chemotherapy despite poor pathologic complete response [5].

This study focuses on identifying the cases of breast cancer at our centre, performing immunohistochemical studies of the basal Cytokeratin CK5/6 in them and studying their expression and correlation with various clinicopathological parameters.

II. MATERIALS AND METHODS

Cases of breast carcinomas diagnosed between the years 2009 and 2014 were included in our study. The study was performed after getting approved by the Institutional Human Ethics Committee (IHEC). A few of the cases were rejected owing to the absence of sufficient clinical information, ER/PR studies or if blocks were unavailable. The requisition form sent by the operating surgeon was used for deriving information such as age, site, nodal status and other gross findings. Hematoxylin and eosin stained slides from representative sections of the breast tumours were used for grading and assessing the histological type of tumour, evidence of lymphovascular invasion, perineural invasion and skin involvement. Immunohistochemical staining for Estrogen Receptor, Progesterone Receptor, Her2neu and CK5/6 was performed on these sections after antigen retrieval in pressure cooker followed by EDTA buffer at an alkaline pH (pH of 9).

The antibody reagent clones were Clone EP1 by DAKO, Clone PgR 636 by DAKO, Anti-v-erbB-2 Clone CB11 by Biogenix and FLEX Monoclonal Mouse Anti-human Cytokeratin5/6 (Clone D5/16B4) for ER, PR, Her2neu and CK5/6 respectively. A two stage process involving binding of primary antibody to the targeted epitope; second step by identifying a secondary antibody bound to a dextran polymer with the help of horseradish peroxidase enzyme attached to a chromogen.

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The various parameters analyzed were age, histological type, size of the tumour, grade, skin, lymphovascular and perineural invasions, number of axillary lymph nodes showing metastasis and staining properties of ER,PR, Her2neu and CK5/6.

Based on studies conducted by Rakha et al [1], Laakso et al [6] an arbitrary scoring system was drawn up for quantifying the expression of CK5/6. (Table: 1)

| Score | Description |
|-------|---|
| 0 | Less than 1 % positivity |
| 1 + | 1-10% tumour cells are positive |
| 2 + | 10-50% tumour cells are positive |
| 3 + | More than 50% tumour cells are positive |

III. RESULTS

44 cases of female breast cancer were under study. The ages ranged between 33 and 67 years. The age group that had the most number of cases was 41 to 50 years. The commonest histological grade in our study was Grade 2, with 24 cases and 9 cases were grade 3(Fig:1).

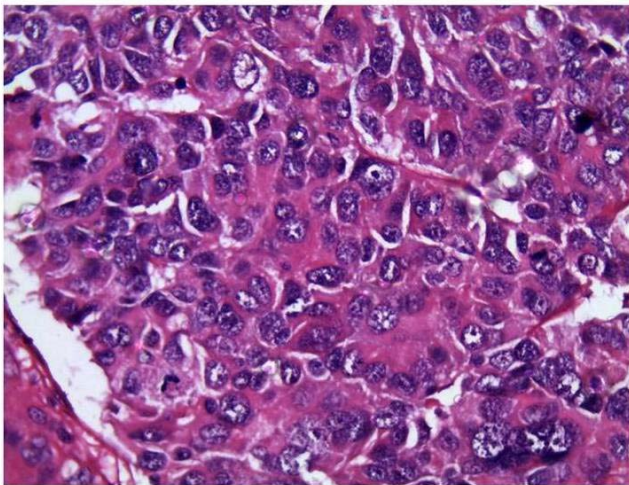


Fig. 1: Invasive ductal carcinoma NOS, H&E 400x

88% of the tumours were of the infiltrating ductal carcinoma, not otherwise specified (NOS). Two cases of micro papillary carcinoma, a case of metaplastic carcinoma and papillary carcinoma were included. Most cases (22) were of sizes between 2cm-5 cm. Lympho vascular invasion was seen in 20 out of 44 cases, with most cases belonging to Grade 2. Perineural invasion (Fig: 2) was seen in only two cases.

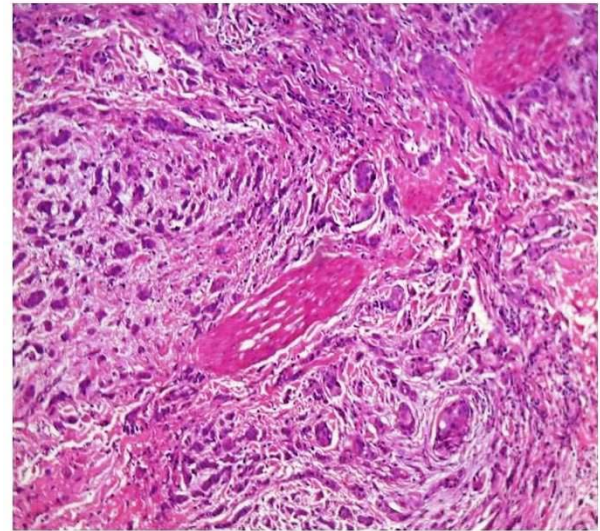


Fig. 2: Invasive ductal carcinoma with perineural invasion, 400x

Estrogen receptor expression was seen in around 45% of cases and 52% of the cases showed Her 2 neu over expression. (Fig 3)

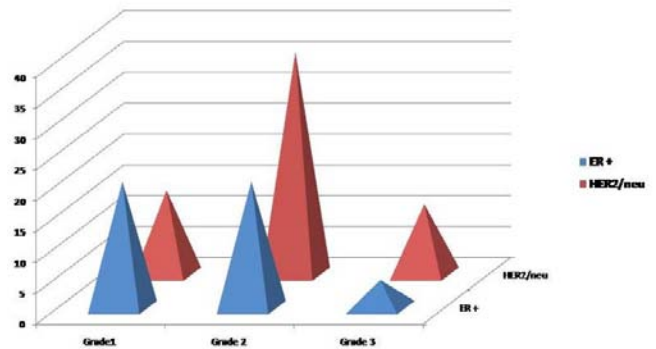


Fig. 3: Histogram showing distribution of ER/Her 2 neu & grade

Size of the tumour statistically correlated with CK 5/6 positivity. Larger tumours had a greater incidence of CK 5/6 positivity with the largest tumour being 13.5 cm in size. (Fig 4)

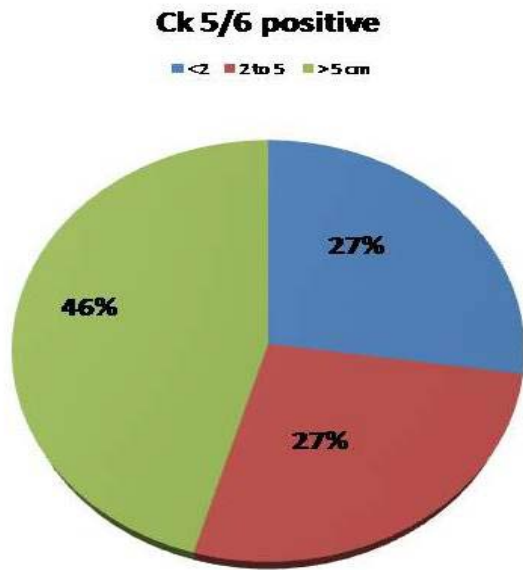


Fig. 4: Pie chart showing CK5/6 positive cases and size of tumours

Correlation between CK 5/6 with invasive and prognostic features was performed and we observed that the tumors with higher grade, lymphovascular, perineural invasion and extensive lymph node metastases showed greater CK5/6 expression than their respective counterparts. (Fig 5)

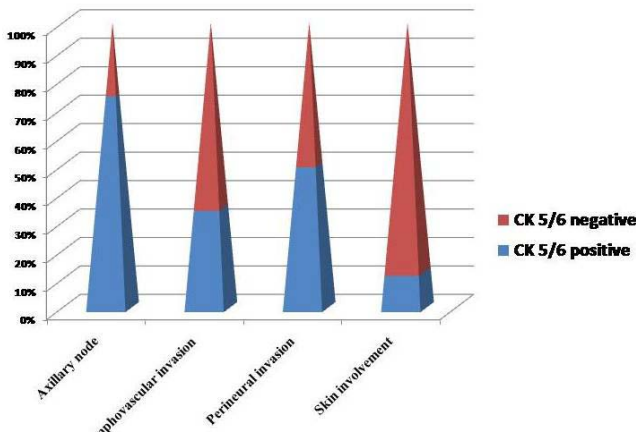


Fig. 5: Histogram showing prognostic parameters vs CK5/6 positivity

IV. DISCUSSION

Breast carcinomas have emerged as the most common malignancy in Indian women. Not only is their incidence high, but the fatality rate of these cases exceeds those of the western population [6]. The cure rates, quality and length of life have improved in these women after the development of targeted therapy. Glass et al [7] observed quantitative and qualitative trends in breast cancer incidence. There has been a tremendous increase, particularly in ER positive tumours. The reason for the exponential rise has been attributed to the use of

post-menopausal hormone replacement therapy and widespread utilization of mammography.

Breast cancers have been sub classified into 4 molecular subtypes.

1. *Luminal A* – High expression of luminal cytokeratins and hormone receptors. ER/PR-positive.HER2/neu-negative.
2. *Luminal B* - Expression of luminal cytokeratins are seen. ER/PR- positive.HER2/neu expression-variably positive. Higher grade and proliferation than Luminal A.
3. *HER2/neu* - Low expression of ER. High expression of HER2/neu and 17q12. ER/PR negative. HER2/neu positive. High grade, TP53 mutations present and higher likelihood of nodal metastasis.
4. *Basal-like* - High expression of basal cytokeratins and epithelial genes. Low expression of hormone receptors. Triple-negative (ER, PR, HER2/neu). TP53, BRCA1 mutations seen.

Treatment response to endocrine therapy is good in 'Luminal type A' tumours. The response in 'Luminal B' tumours is not as satisfactory as in Luminal A tumours. Her 2 neu class of tumours responds to Trastuzumab. The basal like tumours do not responds to endocrine therapy or Trastuzumab.

The subsets of breast carcinoma which are not susceptible to conventional therapy, have a paradigm shift in molecular genetics and immunohistochemical expression. These are the Basal-like breast carcinomas and the Triple Negative Breast Cancers.

The overall percentage of ER positive cases in our study was 45%, lesser when compared with western literature.(Fig 6). This is consistent with findings in a study conducted by Ambroise et al [8], which concluded saying that hormonal expression is lesser in the south Indian population. We noted that 54% of ER positive tumours were node positive and most ER positive neoplasms [9] were less than 2 cm in size.

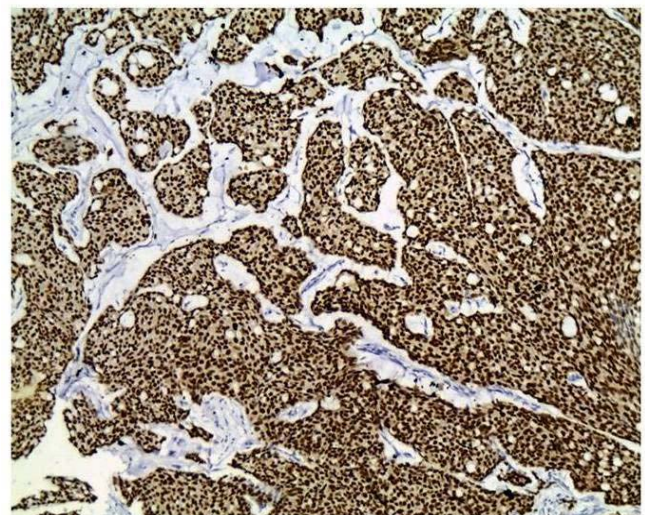


Fig. 6: 100% cells showing strong nuclear staining for Estrogen Receptor

We inferred that due to the large size of the tumours in our study, there was increased nodal metastasis. Almost all the women with ER positive cases belonged to the 35 to 65 year age group.

Normalization technique was introduced for standardization of results and to avoid discordance between immunohistochemistry and FISH results. There is improved accuracy of HER2 studies using a subtraction scoring system in which a signal score of non-neoplastic breast epithelium is subtracted from that of the tumour [10]. Using this system, the proportion of HER2 positive tumours in our study is 63%. (Fig 7). This stresses the need to look into other markers and their routine use in South Indian cohorts.

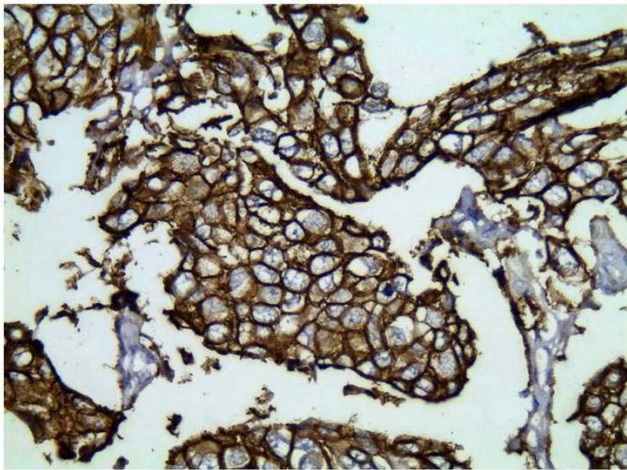


Fig. 7: Cells showing strong and complete membrane staining for Her2neu

Dolle et al [11] inferred that Triple Negative Breast Cancers (TNBCs) are breast cancer subtypes associated with high mortality rate and resistance to hormonal manipulation and Herceptin. Since these tumours are negative for ER, PR and HER2, newer markers are to be identified for this subtype. These tumours have been seen with increased incidence in younger women (aged 45 years or younger). Our study showed close correlation, with the mean age of women with TNBCs being 46 years and nearly a third of the women were 40 or younger.

The purpose of our study was to identify a newer basal marker and observe the expression and clinic-pathological in cases of breast cancer at our centre. The basal marker that we selected for our study was CK 5/6[12]. Clark et al suggested that CK5 is positive in breast progenitor cells, which are believed to be the cell of origin in basal-like breast cancers (Rakha) [1]. In our study, 25% of the cases were basal-like, with all of these tumours falling into either Grade 2 or Grade 3. Thus CK 5/6 was positive in 50 % of TNBCs (Fig: 8). The only case of metaplastic tumour was negative for basal markers.

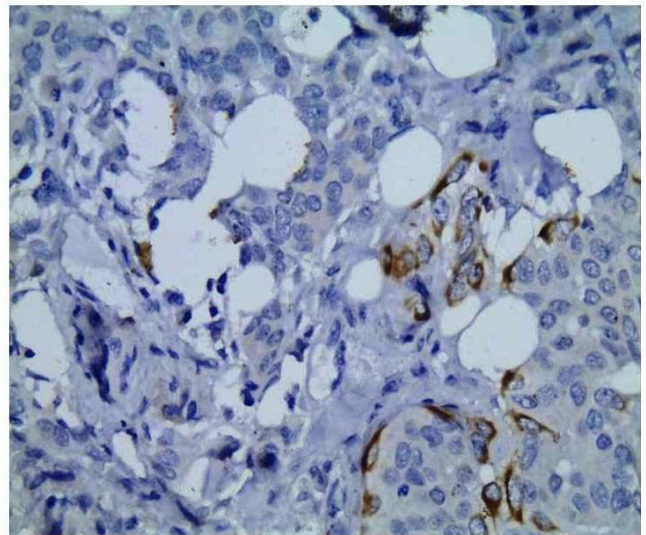


Fig. 8: < 10% cells showing staining with CK5/6 IHC marker, IHC 100 x

Nodal positivity in basal Cytokeratin positive cases in our study was nearly 80%. Vascular emboli were also prominent in these tumours, consistent with their highly invasive nature (Fig: 9). These malignancies are associated with poor prognosis and have a distinctive response to chemotherapy.

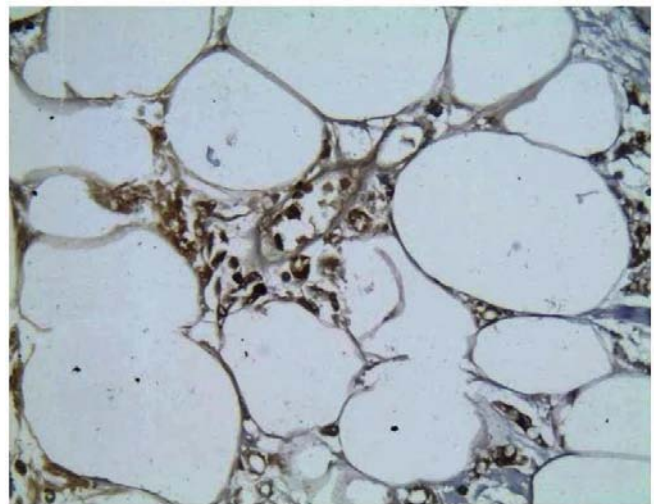


Fig. 9: > 50% cells showing staining with CK5/6 IHC marker, IHC 100 x

Nielson et al [13] observed that a panel consisting of ER, HER2 and CK 5/6 to identify the basal-like subset was useful as this immunohistochemical combination had a 76% sensitivity and 100% specificity rate when compared with genetic microarray analysis.

The conclusions drawn from our study is that CK5/6 positivity was seen in tumours of larger size and higher grades.

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The skilled team of technicians at our histopathology laboratory who helped us with the practical aspects of this study.

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Optimization of Citric Acid Production by Substrate Selection using Gamma Ray Induced Mutant Strain of *Aspergillus Niger*

By Shamima Nasrin, Mesbah Uddin Ansary & Md. Khorshed Alam

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Abstract- The worldwide demand for citric acid is increasing with the rising population and industrialization. The growing demand for citric acid and the need for alternative materials as substrates in the recent years have led to the choice of a novel and economically viable substrate, namely jackfruit (outer portion) and molasses for citric acid biosynthesis. Hydrolysis these substrates with 0.05N HCl followed by fermentation using two isolates of *Aspergillus niger* were investigated for citric acid production under submerged culture condition in a period of 15 days. The products of the microbial metabolism namely residual sugar, total titratable acidity (TTA), citric acid, and biomass contents were determined periodically. Maximum citric acid production was found after 12 days of fermentation for both isolates, namely *Aspergillus niger* CA16, the parent strain and gamma ray induced mutant *Aspergillus niger* 79/20. Citric acid production was found highest in the absence of Prescott salt by *Aspergillus niger* CA16 in mixed fermentation medium which was about 16.35 mg/ml and lowest in jackfruit medium, 12.88 mg/ml at day 12. Whereas in the presence of Prescott salt, lowest citric acid production was also found in jackfruit medium, 7.21 mg/ml and highest in mixed medium, 11.54 mg/ml. In case of the previously isolated gamma-ray induced mutant *Aspergillus niger* 79/20, the yield seems to be higher under similar experimental condition.

Keywords: *aspergillus niger* 79/20, citric acid, titratable acidity.

GJMR-C Classification: NLMC Code: QW 4



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Optimization of Citric Acid Production by Substrate Selection using Gamma Ray Induced Mutant Strain of *Aspergillus niger*

Shamima Nasrin ^α, Mesbah Uddin Ansary ^σ & Md. Khorshed Alam ^ρ

Abstract- The worldwide demand for citric acid is increasing with the rising population and industrialization. The growing demand for citric acid and the need for alternative materials as substrates in the recent years have led to the choice of a novel and economically viable substrate, namely jackfruit (outer portion) and molasses for citric acid biosynthesis. Hydrolysis these substrates with 0.05N HCl followed by fermentation using two isolates of *Aspergillus niger* were investigated for citric acid production under submerged culture condition in a period of 15 days. The products of the microbial metabolism namely residual sugar, total titratable acidity (TTA), citric acid, and biomass contents were determined periodically. Maximum citric acid production was found after 12 days of fermentation for both isolates, namely *Aspergillus niger* CA16, the parent strain and gamma ray induced mutant *Aspergillus niger* 79/20. Citric acid production was found highest in the absence of Prescott salt by *Aspergillus niger* CA16 in mixed fermentation medium which was about 16.35 mg/ml and lowest in jackfruit medium, 12.88 mg/ml at day 12. Whereas in the presence of Prescott salt, lowest citric acid production was also found in jackfruit medium, 7.21 mg/ml and highest in mixed medium, 11.54 mg/ml. In case of the previously isolated gamma-ray induced mutant *Aspergillus niger* 79/20, the yield seems to be higher under similar experimental condition. In absence of Prescott salt highest production of citric acid was found by mutant *Aspergillus niger* 79/20 in mixed fermentation medium which was about 25.87 mg/ml and lowest in jackfruit medium, 22.12 mg/ml at the day 12 which was even higher than that found in case of the parent strain *Aspergillus niger* CA16. In the presence of Prescott salt highest production of citric acid was found in mixed media, 16.35 mg/ml and lowest in jackfruit medium, 13.94 mg/ml which was again higher than that was obtained in case of the parent strain.

Keywords: *aspergillus niger* 79/20, citric acid, titratable acidity.

I. INTRODUCTION

Citric acid is one of the world's largest tonnages of fermentation products. It is widely used in the food beverage industries as an acidifying and flavor-enhancing agent, pharmaceutical, chemical, cosmetic and other industries for applications such as acidulation, antioxidation, flavor enhancement, preservation, plasticizer and as a synergistic agent. The

worldwide demand for citric acid is met by fermentation mainly by the process involving the filamentous fungus *A. niger*. A number of carbon sources may be used for citric acid fermentation. For commercial reasons, the uses of molasses, sucrose or glucose syrups are favored. The use of molasses in particular is desirable because of its low cost availability.

A. niger is capable of producing very high levels of citric acid, about 90% of the theoretical yield from a carbohydrate source. For an efficient citric acid production, the growth of *Aspergillus* in pellet form is desirable and this can be achieved by process optimization. There is a great worldwide demand for citric acid consumption due to its low toxicity compared with other acidulants used mainly in the pharmaceutical and food industries. Global production of citric acid has now reached 1.4 million tones and there is annual growth of 3.5–4.0 % in demand/consumption. A high rate of acidogenesis in *A. niger* is observed only under conditions of high glycolytic metabolism and can be induced by the addition of an excess amount of sucrose or other carbohydrates which induce a high rate of glycolytic catabolism. In this production technique, which is still the major industrial route to citric acid used today, cultures of *Aspergillus niger* are fed on a sucrose or glucose-containing medium to produce citric acid. The source of sugar is corn steep liquor, molasses, hydrolyzed corn starch or other inexpensive sugary solutions. Bangladesh, at present, imported cent percent citric acid from foreign countries. High production depends to a great extent on the strain used and its response to the composition of the medium can show a great deal of variability. Industrial production of this chemical by fermentation using cheap raw materials is helpful in economic development of our country. Keeping in view the future requirements and also the availability of cheap raw material, efforts were made to develop the process for citric acid fermentation, based on our local resources such as molasses from sugar mills and outer portion of jackfruit. So the purpose of present study describes the feasibility of using raw and cheap materials such as molasses and outer portion of jackfruit for citric acid fermentation and to use parent strain CA16 & gamma-ray induced mutants for high citric acid yielding strain 79/20 of *Aspergillus niger*.

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Aspergillus niger is a haploid filamentous fungi and is a very essential microorganism in the field of biology. *A. niger* is cultured for the industrial production of many substances. Various strains of *A. niger* are used in the industrial preparation of citric acid (E330) and gluconic acid (E574) and have been assessed as acceptable for daily intake by the World Health Organization. *A. niger* is important because of its involvement in producing citric acid as well as industrial enzymes, such as amylases, proteases, and lipases. The uses of these enzymes are essential because of its importance for transformation to food enzymes. For example, *A. niger* glucoamylase is used in the production of high fructose corn syrup, and pectinases are used in cider and wine clarification. Glucose oxidase is used in the design of glucose biosensors, due to its high affinity for β -D-glucose. A variety of carbohydrate sources such as beet molasses, cane molasses, sucrose, commercial glucose, starch hydrolysates etc., have been used for citric acid production. Among these, sucrose, cane and beet molasses have been found to be the best choice (Kapoor *et al.*, 1982).

II. MATERIALS AND METHODS

This study was done in the research laboratory of the Department of Biochemistry and Molecular Biology at Jahangirnagar University and at Institute of Food and Radiation Biology, Atomic Energy Research Establishment, Bangladesh during July 2009 and June 2010.

Parent strain *Aspergillus niger* CA16 and mutant strain *Aspergillus niger* 79/20 were first grown on agar slant medium. Each of the properly processed substrates [Molasses, jackfruit (outer portion) and mixed substrates] was hydrolyzed by 0.05 N HCl and filtered which were then used as medium for submerged fermentation. Each substrate were divided into two groups and were fermented separately, one in the presence of Prescott salt and the other in the absence. Each of the groups of the three types of media were further divided into another two subgroups and one of them was inoculated with the parent strain *Aspergillus niger* CA16 and the remaining one was inoculated with the mutant strain *Aspergillus niger* 79/20. All the flasks were then incubated for 15 days in an incubator under same conditions. Fermented media were collected on day 3, 6, 9, 12, 15 and were subjected to estimation of residual sugar, TTA value, citric acid concentration and pH determination.

III. CHEMICAL REAGENTS AND SOLUTIONS

All chemicals and reagents used in this study were of analytical grade. All aqueous solutions were prepared with distilled water. Stock solution of Prescott salt (NH₄NO₃: 2.23g/L, K₂HPO₄: 1.00g/L, MgSO₄.7H₂O: 0.23g/L) used in the media were

prepared 10 times the media concentration and diluted as its normal strength in the experiment.

IV. ANALYTICAL DETERMINATION

At the different stages of fermentation the culture flasks were taken out of the incubator and the medium was collected onto the screw cap test tubes by pipetting and preserved at 0°C. The appropriate amount of sample was used for the estimation of total titratable acidity, citric acid and amount of residual sugar present in the medium after fermentation.

V. DETERMINATION OF TOTAL TITRATABLE ACIDITY (TTA)

Fermented medium (0.25ml) was diluted with 20ml of distilled water and was titrated against 0.1N NaOH solution using 2 to 3 drops of phenolphthalein as indicator. The value obtained was multiplied by 4 and total titratable acidity was expressed as ml of 0.1N NaOH required to neutralize 1ml fermented medium. The titrametric analysis of fermentation of each strain gave an indication of total acidity of the medium. The medium containing high TTA value i.e. higher acid content were then analyzed spectrophotometrically.

VI. ESTIMATION OF CITRIC ACID FROM FERMENTATION MEDIUM

Citric acid was estimated spectrophotometrically by the reference method of Marier and Boulet (1958). Citric acid forms a color complex of polyvinyl keto-anhydridepolymer when it reacts with acetic anhydride and pyridine which can be estimated spectrophotometrically (Auterhoff and Schwingel, 1975). Following the growth of the organism aliquots of the medium were diluted so as to have concentration in the range of 25 to 200 μ g per ml (approximately) of citric acid.

VII. ESTIMATION OF RESIDUAL SUGAR

Before inoculation and after completion of fermentation, samples were collected for initial and residual sugar estimation, respectively.

Following the fermentation, amount of residual sugar in the medium was determined by diluting the aliquots of the medium so as containing sugar concentration range of 25-200 μ g per ml.

Initial and residual sugar of the medium was determined spectrophotometrically by anthrone method (Morse, 1947) using anthrone as the coloring agent with sucrose as standard.

VIII. RESULTS

a) Estimation of residual sugar at different periods of citric acid fermentation

The residual sugar concentration was different in various media during citric acid fermentation by

Aspergillus niger parent strain CA16 and mutant strain 79/20. Prescott salt was also found to have an effect on sugar concentration during citric acid fermentation. In the presence of Prescott salt, residual sugar found in the molasses fermentation medium by *Aspergillus niger* CA16 on day 3, 6, 9, 12 and 15 was 34.81, 26.38, 21.13, 17.75 and 14.64 mg/ml respectively and that in the absence of Prescott salt residual sugar found was 35.19, 30.25, 23.75, 15.23 and 10.92 mg/ml respectively. Lowest amount of sugar was found at Day 15 on molasses fermentation medium by *Aspergillus niger* CA16 in the absence of Prescott salt (Figure- 15). Residual sugar found in the molasses fermentation medium by *Aspergillus niger* 79/20 on day 3, 6, 9, 12 and 15 was 35.64, 29.62, 21.98, 16.20 and 11.51 mg/ml respectively, in the presence of Prescott salt and 40.12, 34.41, 30.13, 25.55 and 17.51 mg/ml respectively in the absence of Prescott salt. At Day 15 lowest amount of residual sugar was found on molasses fermentation medium by *Aspergillus niger* 79/20 in the presence of Prescott salt which is higher than that found by *Aspergillus niger* CA16 in the absence of Prescott salt (Figure-14 & 15).

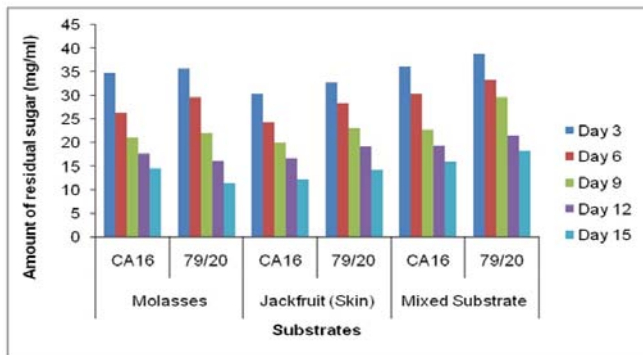


Figure 14: Amount of Residual Sugar at different days of fermentation in various substrate (with Prescott salt) by *A. niger* CA16 and mutant strain *A. niger* 79/20.

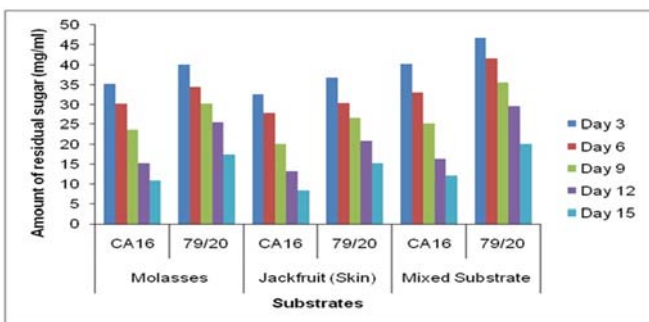


Figure 15: Amount of residual sugar at different days of fermentation in various substrates (Without Prescott salt) by *A. niger* CA16 and mutant strain *A. niger* 79/20.

When *Aspergillus niger* CA16 used jackfruit (outer portion) as fermentation medium, the residual sugar found on day 3, 6, 9, 12 and 15 in presence of Prescott salt was 30.34, 24.34, 20.04, 16.78 and 12.32

mg/ml respectively and in the absence of Prescott salt was 32.55, 27.79, 20.13, 13.24 and 8.40 mg/ml respectively. Lowest amount of residual sugar was found in the absence of Prescott salt on the day 15 on the jackfruit (outer portion) fermentation medium by *Aspergillus niger* CA16 (Figure-15). In presence of Prescott salt, concentration of residual sugar on jackfruit (outer portion) fermentation medium by the mutant fungus *Aspergillus niger* 79/20 was 32.69, 28.43, 23.12, 19.24 and 14.34 mg/ml on day 3, 6, 9, 12 and 15 respectively. On the other hand, the residual sugar concentration was 36.73, 30.29, 26.51, 20.91 and 15.12 mg/ml during same incubation periods in the absence of Prescott salt. Lowest amount of residual sugar was found in the presence of Prescott salt on the day 15 in the jackfruit fermentation medium by *Aspergillus niger* 79/20 which is higher than that found by *Aspergillus niger* CA16 in the absence of Prescott salt (Figure-14 & 15).

On the mixed fermentation medium, residual sugar found in presence of Prescott salt was 36.14, 30.47, 22.81, 19.33 and 15.94 mg/ml respectively and in the absence of Prescott salt was 40.23, 33.021, 25.17, 16.35 and 12.13 mg/ml respectively on day 3, 6, 9, 12 and 15 by *Aspergillus niger* CA16. Lowest amount of residual sugar was found in the absence of Prescott salt on day 15 in mixed fermentation medium by *Aspergillus niger* CA16 (Figure-15). In presence of Prescott salt, residual sugar concentration was 38.81, 33.32, 29.62, 21.64 and 18.29 mg/ml respectively in mixed fermentation medium by *Aspergillus niger* 79/20 on day 3, 6, 9, 12 and 15. On the other hand, the residual sugar concentration in the absence of Prescott salt was 46.64, 41.69, 35.46, 29.60 and 20.12 mg/ml respectively on the same respective days. Lowest amount of residual sugar was found in the presence of Prescott salt on the day 15 in the jackfruit fermentation medium by *Aspergillus niger* 79/20 which is higher than that found by *Aspergillus niger* CA16 in the absence of Prescott salt (Figure-14 & 15).

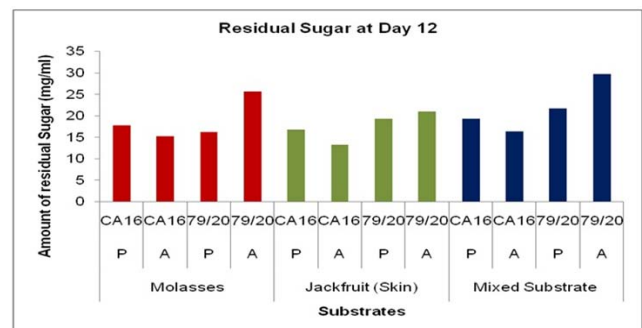


Figure 16: Amount of residual sugar at day 12 of fermentation in different substrates (with and without Prescott salt) by *A. niger* CA16 and mutant strain *A. niger* 79/20. Here, P & A indicates presence & absence of Prescott salt respectively.

On day 12, the residual sugar found on each of the three types fermentation media by the parent strain *Aspergillus niger* CA16 were somewhat lower in the absence of Prescott salt. On the other hand the residual sugar found on the same types of media by the mutant strain *Aspergillus niger* 79/20 were comparatively lower in the presence of Prescott salt on day 12 (Figure- 16).

b) Estimation of TTA values at different period of citric acid fermentation

Total titratable acidity (TTA) of different fermented media were determined after different incubation periods during citric acid fermentation by *Aspergillus niger* parent strain CA16 and mutant strain 79/20. In each case the TTA values were found to increase gradually with the increase in incubation periods from day 3 and picked on day 12 and then started to decrease (day 15) [(Figure-17, 18 & 19) and (Appendix-II & V)].

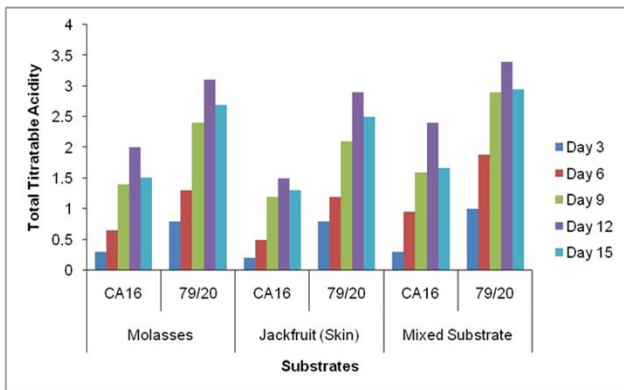


Figure 17: TTA values at different days of fermentation in different substrates (with Prescott salt) by *Aspergillus niger* CA16 and mutant strain *Aspergillus niger* 79/20.

On the molasses fermentation medium, TTA value obtained in the presence of Prescott salt by *Aspergillus niger* CA16 on day 3, 6, 9, 12 and 15 was 0.30, 0.65, 1.40, 2.0 and 1.51 respectively and in the absence of Prescott salt was 0.4, 0.8, 1.8, 2.98 and 2.35 respectively. Highest TTA value for *Aspergillus niger* CA16 was found in the absence of Prescott salt on 12 day. TTA value for mutant strain *Aspergillus niger* 79/20 on the same medium and on the same incubation periods was 0.8, 1.3, 2.4, 3.1 and 2.7 respectively in the presence of Prescott salt and 1.1, 2.4, 3.5, 4.9 and 4.3 respectively in the absence of Prescott salt. The highest TTA value was obtained in the absence of Prescott salt for the mutant strain *Aspergillus niger* 79/20 which was higher than that found in case of parent strain *Aspergillus niger* CA16 (Figure-17 & 18).

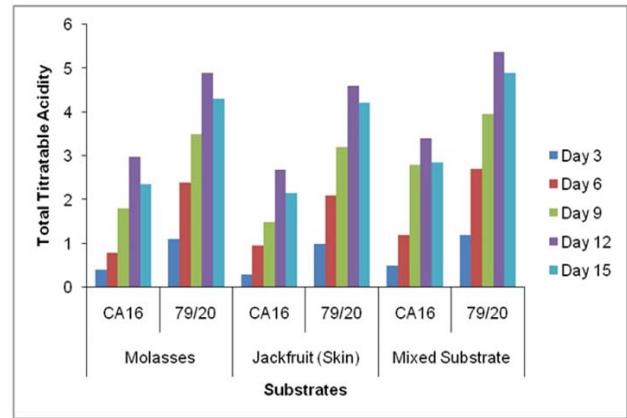


Figure 18: TTA values at different days of fermentation in different substrates (without Prescott salt) by *Aspergillus niger* CA16 and mutant strain *Aspergillus niger*.

In the presence of Prescott salt, TTA value found in the jackfruit (outer portion) fermentation medium by *Aspergillus niger* CA16 on day 3, 6, 9, 12 and 15 was 0.2, 0.5, 1.2, 1.5 and 1.3 respectively and that in the absence of Prescott TTA value found was 0.3, 0.96, 1.5, 2.68 and 2.16 respectively. Highest TTA value was found at Day 12 on jackfruit fermentation medium by *Aspergillus niger* CA16 in the absence of Prescott salt. When *Aspergillus niger* 79/20 was the strain used for fermentation on jackfruit medium, the TTA value found in the presence of Prescott salt on day 3, 6, 9, 12 and 15 was 0.8, 1.2, 2.1, 2.9 and 2.5 respectively and in the absence of Prescott salt was 1.0, 2.1, 3.2, 4.6 and 4.22. TTA value was highest at day 12 in the absence of Prescott salt for *Aspergillus niger* 79/20 which was higher than that obtained for *Aspergillus niger* CA16 (Figure-17 & 18).

TTA value obtained on day 3, 6, 9, 12 and 15 was 0.3, 0.95, 1.6, 2.4 and 1.67 respectively in the presence of Prescott salt and in the absence of Prescott salt was 0.5, 1.2, 2.8, 3.4 and 2.85 respectively when the parent strain *Aspergillus niger* CA16 was allowed to ferment the mixed medium. Highest TTA value found in the absence of Prescott salt for *Aspergillus niger* CA16 grown on mixed fermentation medium. When the same medium was fermented by *Aspergillus niger* 79/20, the TTA value obtained in the presence of Prescott salt on day 3, 6, 9, 12 and 15 was 1.0, 1.89, 2.9, 3.4 and 2.95 respectively and in the absence of Prescott salt the TTA value was 1.2, 2.7, 3.95, 5.38 and 4.89 respectively. Once again the highest TTA value was obtained for *Aspergillus niger* 79/20 on day 12 in the absence of Prescott salt which was higher than that obtained in case of *Aspergillus niger* CA16 (Figure-17 & 18).

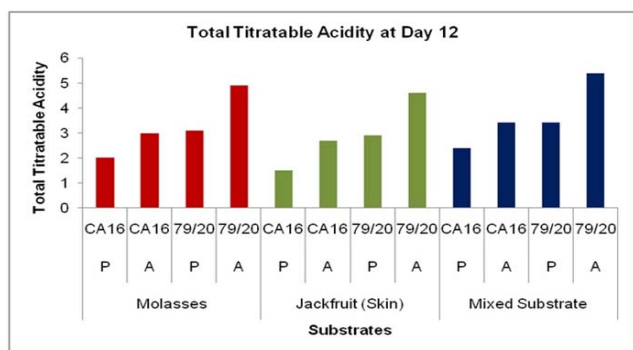


Figure 19: Total Titratable Acidity at day 12 of fermentation in different substrates (with and without Prescott salt) by *A. niger* CA16 and mutant strain *A. niger* 79/20. Here, P & A indicates presence & absence of Prescott salt respectively.

These results showed TTA value was comparatively higher in the absence of Prescott salt for all the three types of media and for each of the stain. Throughout the incubation period the TTA value was highest in case mixed fermentation medium followed by molasses and jackfruit fermentation medium. Again, fermentation by *Aspergillus niger* 79/20 resulted in a comparatively higher TTA value than by *Aspergillus niger* CA16 both in the presence and absence of Prescott salt (Figure 19).

c) Estimation of citric acid accumulation at different period of citric acid fermentation

Accumulation of citric acid at different incubation periods on different media followed a very similar pattern as was seen in case of TTA value. Citric acid concentration was also different on different incubation periods with various fermentation media by the parent strain *Aspergillus niger* CA16 strain and the mutant strain 79/20. Citric acid concentration was found to increase gradually with the increase of incubation period and maximum citric acid concentration was found on day 12 in case of each of the three media. Finally, citric acid concentration was found to decrease at day 15 [(Figure-20, 21 & 22) and (Appendix-III & VI)].

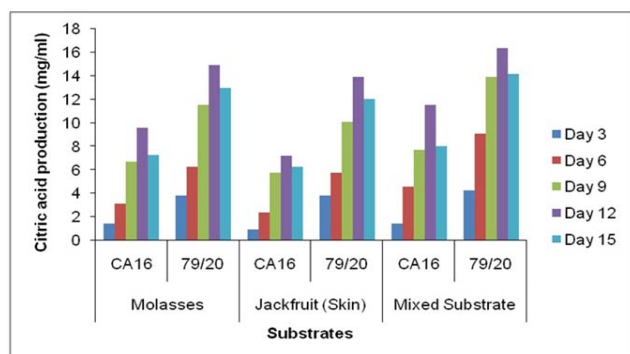


Figure 20: Citric acid accumulation at different days of fermentation in different substrates (with Prescott salt) by *A. niger* CA16 and mutant strain *A. niger* 79/20.

Citric acid concentration obtained on day 3, 6, 9, 12 and 15 was 1.44, 3.13, 6.73, 9.62 and 7.26 mg/ml respectively in the presence of Prescott salt and in the absence of Prescott salt was 1.92, 3.85, 8.65, 14.33 and 11.29 mg/ml respectively when the parent strain *Aspergillus niger* CA16 was allowed to ferment the molasses medium. Highest citric acid concentration found at day 12 in the absence of Prescott salt for *Aspergillus niger* CA16 grown on molasses fermentation medium. When the same medium was fermented by *Aspergillus niger* 79/20, the citric acid concentration obtained in the presence of Prescott salt on day 3, 6, 9, 12 and 15 was 3.85, 6.25, 11.54, 14.90 and 12.98 mg/ml respectively and in the absence of Prescott salt the citric acid concentration was 5.29, 11.54, 16.83, 23.56 and 20.67 mg/ml respectively. Once again the highest citric acid concentration was obtained for *Aspergillus niger* 79/20 on day 12 in the absence of Prescott salt which was higher than that obtained in case of *Aspergillus niger* CA16 (Figure-20 & 21).

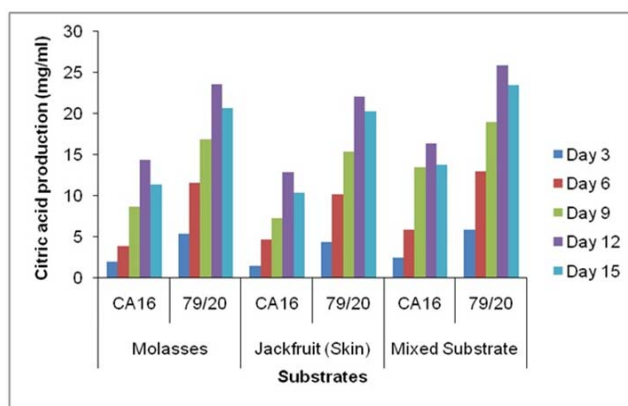


Figure 21: Citric acid accumulation at different days of fermentation in different substrates (without Prescott salt) by *A. niger* CA16 and mutant strain *A. niger* 79/20.

On the jackfruit (outer portion) fermentation medium, citric acid concentration obtained in the presence of Prescott salt by *Aspergillus niger* CA16 on day 3, 6, 9, 12 and 15 was 0.96, 2.4, 5.77, 7.21 and 6.25 mg/ml respectively and in the absence of Prescott salt was 1.44, 4.62, 7.21, 12.88 and 10.38 mg/ml respectively. Highest citric acid concentration for *Aspergillus niger* CA16 was found in the absence of Prescott salt on 12 day. Citric acid concentration for mutant strain *Aspergillus niger* 79/20 on the same medium and on the same incubation periods was 3.85, 5.77, 10.09, 13.94 and 12.02 mg/ml respectively in the presence of Prescott salt and 4.29, 10.09, 15.38, 22.12 and 20.29 mg/ml respectively in the absence of Prescott salt. The highest citric acid concentration was obtained in the absence of Prescott salt for the mutant strain *Aspergillus niger* 79/20 which was higher than that found in case of parent strain *Aspergillus niger* CA16 (Figure-20 & 21).

In the presence of Prescott salt, citric acid concentration found in the mixed fermentation medium by *Aspergillus niger* CA16 on day 3, 6, 9, 12 and 15 was 1.44, 4.57, 7.69, 11.54 and 8.03 mg/ml respectively and that in the absence of Prescott citric acid concentration found was 2.40, 5.77, 13.46, 16.35 and 13.70 mg/ml respectively. Highest citric acid concentration was found at Day 12 on mixed fermentation medium by *Aspergillus niger* CA16 in the absence of Prescott salt. When *Aspergillus niger* 79/20 was the strain used for fermentation on mixed medium, the citric acid concentration found in the presence of Prescott salt on day 3, 6, 9, 12 and 15 was 4.29, 9.09, 13.94, 16.35 and 14.18 mg/ml respectively and in the absence of Prescott salt was 5.77, 12.98, 18.99, 25.87 and 23.51. Citric acid concentration was highest at day 12 in the absence of Prescott salt for *Aspergillus niger* 79/20 which was higher than that obtained for *Aspergillus niger* CA16 (Figure-20 & 21).

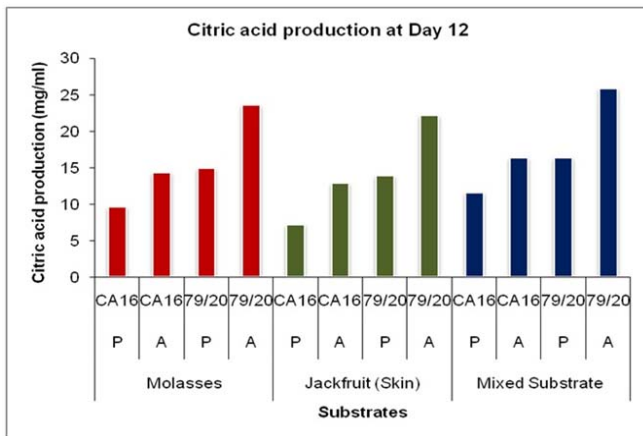


Figure 22: Citric acid accumulation at day 12 of fermentation in different substrates (with and without Prescott salt) by *A. niger* CA16 and mutant strain *A. niger* 79/20. Here, P & A indicates presence & absence of Prescott salt respectively.

These results showed citric acid concentration was comparatively higher in the absence of Prescott salt for all the three types of media and for each of the strain. Throughout the incubation period the citric acid concentration was highest in case mixed fermentation medium followed by molasses and jackfruit fermentation medium. Again, fermentation by *Aspergillus niger* 79/20 resulted in a comparatively higher citric acid accumulation than by *Aspergillus niger* CA16 both in the presence and absence of Prescott salt (Figure-22).

Fermentation of citric acid for commercial production is dependent on many factors like quality of strains, nutritional composition of the media, environmental conditions, deficiency of manganese and other metals, pH, temperature and dissolved oxygen tension (Moyer, A. J. 1953). Usually, *Aspergillus niger* gives the best yield at around 25-28°C. Increase in

incubation period resulted in the increased citric acid production. A lower concentration of sugar leads to lower yield of citric acid as well as accumulation of oxalic acid (Kovats, 1960). But the use of wild type strain of *Aspergillus niger* is not cost effective. So, high yielding strains were searched which will give the best yield at around the room temperature. The superior strains *Aspergillus niger* CA16 and gamma ray induced mutants *Aspergillus niger* 79/20 seem to have fulfilled the requirement. Thus these strains can be conveniently exploited for the production of citric acid from cane molasses, jackfruit (outer portion) and a mixture of the two substrates.

From the findings of this study it is clearly suggested that both fermentation medium and Prescott salt have a considerable effect on the production of citric acid. Among the media used in this study, the mixed fermentation medium was found to be most suitable for citric acid production followed by molasses and jackfruit (outer portion) media. Another important finding of the present study was that Prescott salt was found to have a negative effect on the citric acid production by the either strains of *Aspergillus niger*. Again the gamma-ray induced mutant strain, *Aspergillus niger* 79/20 had a yield efficiency more than that of the parent strain *Aspergillus niger* CA16 and thus considered superior to the parent strain *Aspergillus niger* CA16. Thus as far as citric acid production is concerned the mixed medium in the absence of Prescott salt is the most suitable medium and the gamma ray induced mutant strain *Aspergillus niger* 79/20 is the preferred organism.

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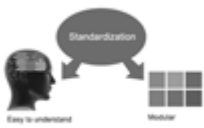
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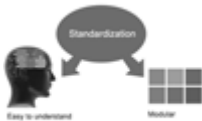


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