Polymerase Chain Reaction for Detection and Genotyping of Molluscum Contagiosum Virus in Diyala Province

Mohammed Khalifa AL –Azawi (Assist. Prof .Dr)

Abstract

Background: Molluscum contagiosum virus (MCV): is a viral skin infection which may infect themucous membrane and skinoccasionally, It is caused by Molluscipox virus from family Poxviridae. Molluscum contagiosumvirus (MCV) was first described and later assigned its name by Bateman in the beginning of the nineteenth century.

Aim: This study was done to confirm the clinical diagnosis of MCV by laboratory test through using PCR assay andto know the predominate type of MCV that found in Diyala Province.

Patients, and methods:The present study was conducted for the period from1 November 2011 to 30 April of 2012 in outpatient clinic of Baquba Teaching Hospital in Baquba city.

The study aimed to confirm the clinical diagnosis of MCV by laboratory test through using PCR assay and to know the domain subtype of MCV that found in Diyala province
Sixty (60) patients were diagnosed with clinical lesions of MCV on different areas of the body, their age ranged from(1-80 years) including 40(66.7%) males and 20(33.3%) females, and the lesion samples were taken and examined by PCR

Results: After testing by PCR, 51(85%) of patients gave positive results for MCV,30(58.8%) patients gave positive results for MCV type 1(26.7%) and 2 (73.3). The results showed 23(45.1%) with age group (31-40 years), included 36(70.6%) were males and 15(29.4%) females, no significant difference showed between the MCV infection and either the sex or age .

The results revealed that MCV type 2 was more prevalent 22(73%) compared with MCV type 1(26.7) ,most of type 2 (73.3) infected males 14(46.5%) , and found in age group (31-40 years),while the MCV type 1 was equally affecting males and females , consisted of (100%) in age (≤10 years) ,with significant difference recorded between the types and age, but no significant difference between the types and the sex**.**

Conclusions: 85%of examined patients with MC showed postive PCR results for MCV prevalence of MCV type 1 with high in children of age group (≤10) .MCV type 2 was predominately seen in (31-40) patient age group.

key word : Molluscum Contagiosum Virus, PCR, Subtype.

College of Science/ Diyala University/ Diyala/ Iraq.

Introduction

Molluscum contagiosum (MC): is a viral skin infection which may infect the mucous membrane occasionally. It is caused by Molluscipox virus from family Poxviridae. Molluscum contagios umvirus (MCV) was first described and later assigned its name by Bateman in the beginning of the nineteenth century cited by [1]

In 1841 Henderson and Paterson described the intracytoplasmic inclusion bodies, now known as molluscum or Henderson-Paterson bodies.In the early twentieth century, Juliusberg, Wile, and Kingery were able to extract filterable virus from lesions and show transmissibilitycited by [2].

MCV has no animal reservoir, infecting only humans and there are four types of MCV, MCV-1,MCV-2,MCV-3 and MCV-4. MCV -1 was the most prevalent predominantly seen in children and MCV -2 was seen usually in adults and often sexually transmitted [3].

The diagnosis of MCV was usually done clinically. They need for laboratory diagnosis of MCV was speculative,because a spontaneous healing was observed in cases where no underlying immune defect is present, the disease was considered as a self-limiting condition. However, there were several lines of reasoning where medical intervention and treatment might be beneficial. Though molluscum cannot be cultured in the laboratory.Histological examination of a curetted or biopsied lesion can also used in the diagnosis in cases that are not clinically clear. The thick white central core can be expressed and smeared on a slide and left unstained or stained with Geimsa or Gram stains to demonnnstrate the large brick-shaped inclusion bodies. Electron microscopy has also been used to demonstrate MCV structures.

Immunohistochemical methods' using a polyclonal antibody allows recognition of molluscum contagiosum virus in fixed tissue. In-situ hybridization for MCV DNA has also been utilized [4,5].

The best option for the definitive diagnosis of MCV was PCR-based assays. An additional benefit of molecular diagnosis was the results provide information about the type of the infecting molluscum strain. No molecular data have been reported in the literature regarding prevalence of MCV types in Iraq . Thus, in this study we attempted to document the feasibility of DNA amplification-based assay in laboratory.The aims of this study To confirm the clinical diagnosis of MCV by laboratory test through using PCR assay, and to know the predominate type of MCV that found in Diyala Province.

Patients and Methods
A.Collection of samples:-

This study was conducted in outpatient Clinic of Dermatology of Baquba Teaching Hospital as across section study including all patients attending in the period between 1 November 2011 to 3o April 2012. The collection of patients sample was done in dermatology unit after diagnosis by dermatologists the cases and sample was submmited to PCR diagnosis.

The demographic information include age, sex, address, educational status, and last the number and the distribution of lesions present was recorded.

The lesion from each patient was curetted and placed in 5 ml phosphate buffered saline, pH 7.1, and immediately transported to the laboratory. The samples were stored at -37˚C until the extraction of the DNA.

Conventional Polymerase chain reaction was used to detect the Molluscum contagiosum virus, and resection enzyme Bam.HI to type of virus . Sixty (60) samples

were selected depending on the size of lesions of the patient, with lesion not less than 30mg depended on method as described by geneaid compony

B. DNA Extraction:-

Sixty samples were selected depending on their size and after neglecting the PBS, the DNA extraction and purification as instructed by the Geneaid company was done.

C. Primer selection:

Table (1) showed two sets of primers that were used in the study as suggested by [6].

Table (1): primers used in the study.

Set No.	Primer	No .of bp.	Company
1	F1(5-GGCGCGTAGCCGAGCGG-3) R1(5 CTTCCGGGCTTGCCGCCGGGCAG-3)	393-bp	Bioneer
2	KU (5-GGAGGAGTGCCCATCAAGAAT-3) OR (5-GCTTTTCAGTTTTTGTGCGA-3)	575-bp	Bioneer

NO. bp: Number of base pair

The first primers F1 and R1 amplify 393-base pair (bp) portion of p43K polypeptide from MCV genome whereas KUF and OR primers amplify 575-bp-long region from p43K polypeptide of MCV genome. KU and OR primers amplified region containing BamH1 restriction site in sub-type genome of MCV, thus allowing accurate subtyping of the infecting strain.

D.Polymerase Chain Reaction (PCR)

Conventional PCR was the method that applicated on our samples to obtain result. Doubled polymerization process have been performed twice , first PCR to use a primer of first set (F1 and R1) which was used for the diagnosis of molluscum contagiosum and saw any positive or negative lesions, the second set another detection to MCV and find out with kind of molluscum contagiosum was prevalent by using restricted enzyme Bam.H1 after amplified region allowing to digestion. Doubled amliphcation had already been done by (7) with simple modification .

1-The first thermo cycle PCR process include :-

1-Two micro liters of isolated DNA was added to 0.2 of a PCR Pre mix. .PCR Pre mix.kit was selected from bioneer (bioneer/ korea).

2- This mixture(Table 3.5.) containing 10 μM Tris-HCl (pH 8.3), 30 μM KCl, 1.5 mM MgCl$_2$,each deoxynucleoside triphosphate at a concentration of 250 μM,1U of Taq polymerase, 30 pmol (picomol) of primersF1 and R1,and the mixture was complete to 20 micoleter Deionize distill water (D.D water) .

3-The samples were used with a thermal phases involving initial denaturation at 95°C for 1 min and 40 cycles consisting of denaturation at 95°C for 1mint , annealing at 58°C for 1 min, and extension at 72°C for 1 min, after complete the thermo cycle, extension at 72°C for 5 mint and finally hold the reaction at 4°Cfor 5 mint .

4- The amplification reactions were visualized on a 1.5 % agarose.

2.B.Asecond PCR amplification and Bam.HI digestion of amplified products:-

1-Three microliters of isolated DNA was added to 25µl of a Green Master Mix ,PCR reaction kit was selected from the (promega /USA).

2-This mixture containing 10 m MTris-HCl (pH 8.5), 3 mM $MgCl_2$, each deoxynucleoside triphosphate(dATP, dGTP, dCTP, dTTP) at a concentration of 400 µM, 1.25 U of Taq polymerase, 30picomol(pmol) of primers KuF and OR1, and the mixtures was completed to 50 microliter Deionized distilled water (D.D water) .

3-The samples was dised with a thermal phases involving initial denaturation at 95°C for 1 min and 40 cycles consisting of denaturation at 95°C for 1mint , annealing at 58°C for 1 min,and extension at 72°C for 1 min, after complete the rmocycleextention at 72°C for 5 mintand, hold the reaction at 4°Cfor 5 mint .

4- The amplification reactions were visualized on a(2%) agarose gel.

3.1Ethanol Precipitation Of DNA :

Ethanol precipitation Of DNA carried out according to the method of.(8) the salt concentration of the viral DNA samples was balance by addition of ($MgCl_2$) to final concentration of 0.01M and ($NaCl_2$)to final concentration 0.2M . The DNA sample put in to small volume (300) in eppendr of tube. Three volumes of cold (-20°C) absolute ethanol were added to one volume of salt –adjusted DNA sample . The content mixed gently by using micropipette. The DNA ethanol mixture was then kept at (-20°C) overnight and the precipitation DNA was pelleted by centrifugation at 10,000 RPM(Rondom Per Cycle) for 30 minute in eppendr of centrifuge at 4 ° C . The supernatant was gently aspirated and the pellet was re suspended in cold ethanol 70% ethanol . The DNA suspension was centrifuged as above and the pellet was drain in temperature room before the pellet was resuspended in TE buffer(PH 7.8)

4.Enzyme and Buffer :-

One restriction endonuclease enzyme were obtained from promegacompany /USA.

That enzyme was Bam.H1 .
Bam. H1. consist of (Restriction Enzyme 10X Buffer, Acetylated BSA, 10µg/µl.

5.Digestion :-

About 40µl of purified DNA were ethanol precipitation ,pelleted   and draind as described previously .The DNA digestion with Bam.H1 Restriction Enzyme, 10u/µl).

1. In a sterile tube, assemble the following components in the order listed below.

Table (2): Method of digestion by Bam.H1.

Component	Volume
Sterile, de ionized water	16.3µl
Restriction Enzyme 10X Buffer	2µl
Acetylated BSA, 10µg/µl	0.2µl
DNA, 1µg/µl	1.0µl
Mix by pipe ting, then add:-	
Restriction Enzyme, 10u/µl	0.5µl
Final volume	0.5µl

2.Mix gently by pipe ting, close the tube and centrifuge for a few seconds in a micro centrifuge. Incubate at the enzyme's optimum temperature for 1.5-2 hours.

3.Add loading buffer to a 1X final concentration and proceed to gel analysis(1.2% final concentration of agrose gel).in volt 60and 90 mint .

Note:-We don't need Overall night digestions which are usually unnecessary and may result in DNA degradation. As protocol of pro

Results

1.Detection of Molluscum contagiosum virus (MCV) and typing

Fifty one51(85%) of patients skin lesions gave positive result for   MCV, and 9 (15%) gave negative result as in (Table3), fig (1).

Thirty (58.8%) gave positive result for MCV typing 1 and 2 , 21(41.2%) gave negative result ,as in (Table4),fig (2).

Table (3): Detection of MCV in patients samples by PCR technique.

Result	No. of patients	%
Positive	51	85
Negative	9	15
Total	60	100

Table (4): Typing of MCV type 1, 2 in patients samples by PCR technique(n=51).

Result	No. of patient	%
Positive	30	58.8
Negative	21	41.2
Total	51	100

*n= 51: Only positive result from 60 samples that detection byfirst PCR..

2. Distribution of MCV according to following variables:

A. Distribution of MCV according to the age.

The results showed that(23; 45.1%) of patients within age group (31-40 year) positive for MCV ,(11; 21.6%) in the age group (≤10year),(8; 15.7%)in the age group (21-30 year), (4; 7.8%) in the age group (41-50 year),(3; 5.9%) in age group(11-20 year),and(2; 3.9%)in the age group(≥51 year), (Table5).

Table(5): Distribution of MCV according to the age patients:

Age	No. of patients	%
≤10	11	21.6
11-20	3	5.9
21-30	8	15.7
31-40	23	45.1
41-50	4	7.8
≥51	2	3.9
Total	51	100

B. Distribution of MCV according to the sex.

The results showed that MCV was more prevalent in males (36; 70.6%) in comprise with females (15; 29.4%),no significant difference of MCV between both sexes, (Table 6).

Table (6): Distribution of MCV according to the sexes of patients:

Sex	No. of patients	%	P. Value
Male	36	70.6	Ns
Female	15	29.4	
Total	51	100	

Ns: no significant

C. Distribution of patients according to the residence of patients

Most of the patients were from urban area (36; 70.6%) compared with rural group(15; 29.4%) , the difference in   residence was statically significant,(Table7).

2.Detection of MCV typing and effect of sex and age   detected by Bam.H1.

A. Detection type of MCV patients .

MCV type 2 was more prevalent (22; 73.3%)in patients with MCV disease, while MCV type1 represent (8; 26.7%),(Table12).

Table (7): The frequency of types of MCV as detected by Bam. HI. restriction enzyme.

Type of MCV	No. of patients	%	P. Value
MCV type 1	8	26.7	Ns
MCV type 2	22	73.3	
Total	30	100	

Ns: no significant

B. The frequency of MCV type 1 and 2 according to sex.

The results showed that MCV type 2 was more prevalent in males   (14; 46,7%) in camper's to the females patients (8; 26.7%) ,while both males and females were affected equally by MCV type1, no significant difference between the   types of   MCV according to the sex .(Table13)

Table (8): Distribution of MCV type 1 and 2 in relation to sex.

Typeof MCV	Male		Female		Total	%	P. Value
	No.	%	No.	%			
MCV type 1	4	13.3	4	13.3	8	26.7	**Ns**
MCV type 2	14	46.7	8	26.7	22	73.3	
Total	18	60	12	40	30	100	

Ns: no significant

C. Distribution of MCV types according to the age of patients.

The results showed   that MCV type 1 was more prevalent in the children in the age group (≤10year) which were consisted of (8; 26.7%) while all cases   in this group infected by MCV type1 (100%),and MCV type 2was more prevalence in adult age group (31-40 year)(14; 46.5%),   with significant difference was found between the age and the MCV types .(Table14)

**Table(9):** Distribution of MCV types according to the age of patients .

Age	Type 1		Type 2		
	No.	%	No.	%	Total
≤10	8	26.7	0	0	8
11-20	0	0	2	6.7	2
21-30	0	0	2	6.7	2
31-40	0	0	14	46.5	14
41-50	0	0	2	6.7	2
≥51	0	0	2	6.7	2
Total	8	26.7	22	73.3	30
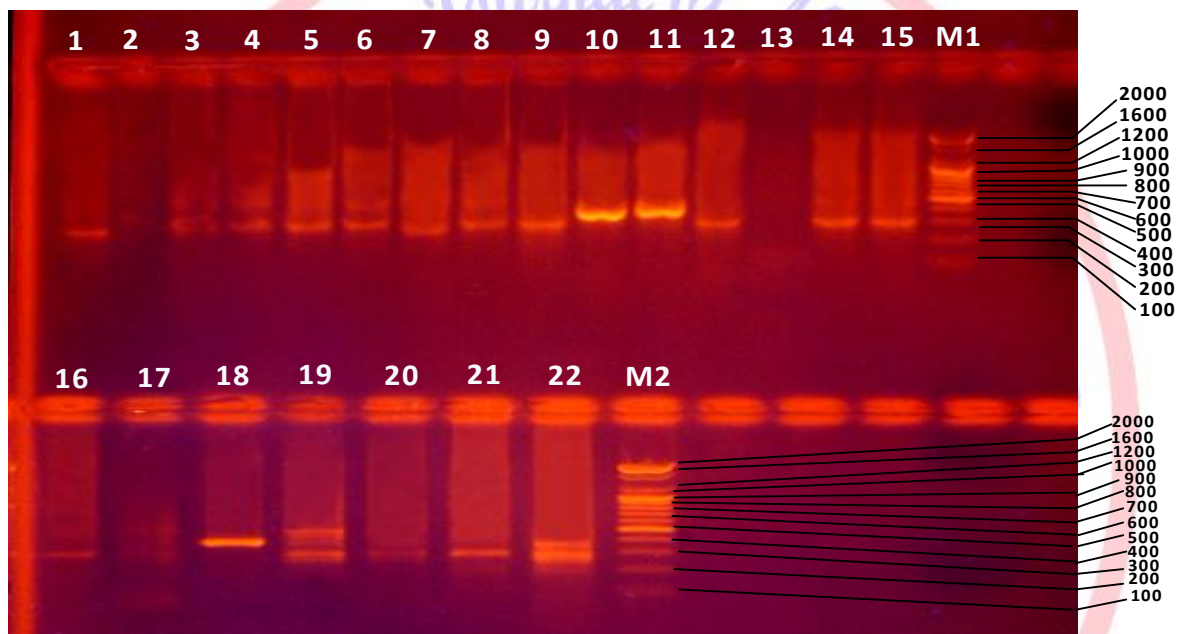
Fig(1): Electrophoresis PCR detection of the extracted DNA from (MCV) lesions as (implicated by positive control 393-bp amplicon) for detection of MCV on agarose gel concentration(1.5%),voltage (60)for (90min.).
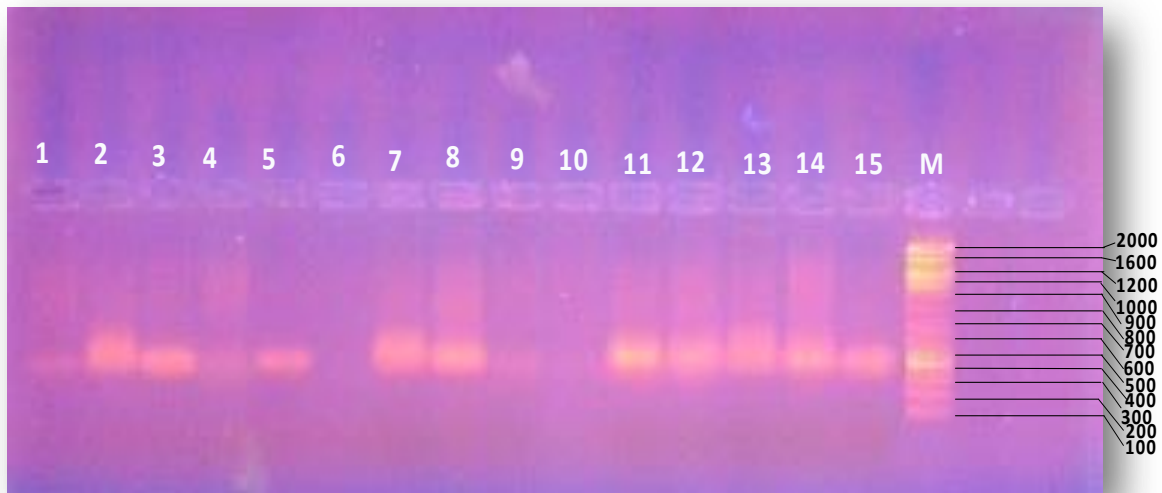
Fig(2): Electrophoresis PCR detection of the extracted DNA from (MCV) lesions as (implicated by positive control 595-bp amplicon) for detection of MCV on agarose gel concentration(1.5%),voltage (60)for (90min.).
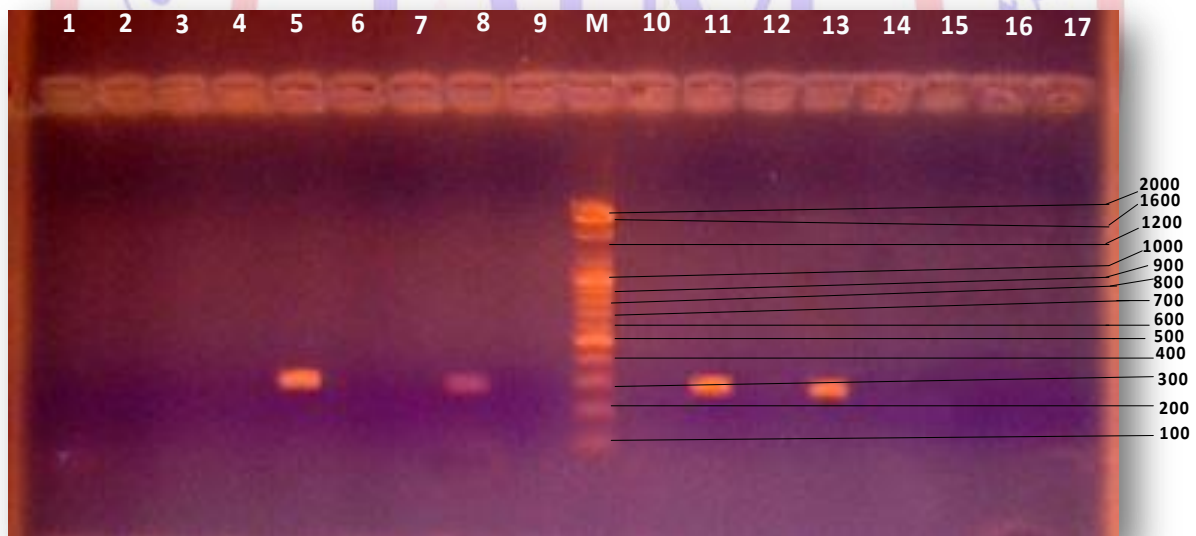
Fig(3): BamH1 digestion of positive 575 bp amplicon for subtyping type -1and type -2 on agarose gel concentration(2%),voltage (60)for (90min.).

Discussion

1. Detection of Molluscum cntagiosum virus (MCV) and its typing.

The present study was disagreement with other reported by researchers (9.6), who found that 100% of cases in their study gave positive results.

This difference in result may be due to mutation happened in this region or may be appeared as a new strain, where type 1 and 2 had more than other subtype (10.11), three

major genomic types with widespread distribution throughout the world had been identified: MCV 1 and its minor variant MCV 1v, MCV 2, and MCV 3, PCR confirm and identify between the two major MCV types (MCV 1 and MCV 2).

This difference in results maybe due to a third type that has not been detected in this study or may be due an error occursin the clinical diagnosis, (Table3.4).

2.Distribution of MCV according to following variables:

A. Distribution of MCV according to age.

This study revealed that most of patients were found in age range (31-40) and that in agreement with the study done by (12). Molluscum contagiosum was most commonly seen in the age group(5-10 years), followed by the age group(1-5 years), then age group (10-14years) and less common in age than-1-years, also in agreement with study in USA reported by(13) in which approximately 80% of the patients was younger than 8 yearsage, and in disagreement with (14) ,who reported that (62%-64%) of patients belonged to ages (11 to 30 years),(Table 5).

B. Distribution of MCV according to the sex.

The study showed that the percentage of MCV was in agreement with reported by Turkish researcher (6) , (40;67.2% in males and 21;32.8% in females)and also in agreement with study reported in Iran researcher (15), (29 ;2.45%) in girls and (46 3.1% in boy) and found these differences were not statistically significant, but in disagreement with study in Egypt in which males represented (42.9%) and females(57.1%),which was statically no significant (16),(Table 6).

This difference in results may be because the lack of educational the lack of attention to health, especially when the emergence of

such diseases is not just of interest because eitisa little knowledge of health matters.

3. Detection of typing of MCV and effect of sex and age.

A. The frequency of MCV type 1 and 2 detected by Bam. HI restriction enzyme.

The present study was in less agreement with Spanishre search reported by (17) ,they found an overwhelming MCV type 1 infection in a population with a ratio146:1 for MCV type1 to type 2, also in disagreement with Turkish study reported by (6) who demonstrated that type1 is the only dominant , and in disagreement with study reported by (18) who found that MCV type in (75%-90% of cases) was the most common, followed by type 2.

This study was in agreement with (19) his results enhance the authors to suggest a possible difference route of transmission for MCV type 2 involving a sexual contact .(20) indicated that the widespread of MCV infection may be due to impairment present of immune system.

However, this variation in result was due to increasing number of adults infection with molluscum contagiosum in recent year sand the figures were more than doubled during the last 10 years, this difference between the incidence of type-1and type-2 maybe as a result of the composition of the community as well as the dominant social relations,, (Table7),fig (.3).

B. The frequency of MCV type 1 and 2 according to sex .

The result was more agreement with (21), where no significant difference was found between type and sex of patients, but in disagreement with that reported by (22) , where the female/male ratio was 1.2:1.

In this study variation in result may be due to the viral virulence which was not affected by the type of sex, therefor the disease affected both sex with little variation

in rate of infection because most of visitors to outpatient clinic of Baquba Teaching hospital from male .(Table8)

C. Distribution of MCV types1 and 2 according to the age.

This study was more agreement with studies reported by (3.23.24)who found respectively showed that type1 is the common cause of the disease in children, with age group (1-10years),the prevalence of MCV type1 was observed the age, type2 higher in the younger age and adults , approximately 80% of the patients were more than 12years old, (Table9).

References

[1] Bateman F.(1953). Molluscum Contagiosum In : Shelley WB, Crissey JT, Editors. Classics In Dermatology. Charles C Thomas:. useful summary of the poxviruses that can zoonotically infect man, which indicates which of these infections are clinically important. springfield (il); p. 20.a

[2] Juliusberg M. Zur- Kenntnis D .(1905).Virus Molluscum Contagiosum. Dtsch Med Wochenschr;31:15989.

[3] Hanson D , Diven DG(2003). Molluscum contagiosum. Dermatol Online Jornal.;9(2):2.

[4] Stulberg DL, Hutchinson AG. Molluscum contagiosumand warts. Am Fam Physician. 2003 Mar;67(6):1233-40.

[5] Bikowski JB. (2004) Molluscum Contagiosum: The Need For Physician Intervention And New Treatment Options. Cutis;73:2002–2006.

[6] Nunez A, Funes JM, Agromayor M, Moratilla M, Varas AJ, Lopez- Estebaranz JL, *et al*. (1996) .Detection and typing of molluscum contagiosum virus in skin lesions by using a simple lysis method and polymerase chain reaction. Journal of Medecine Virol;50:342–349.

[7] Yunus S, Ahmet K, Aykut O, Yasemin B, and Mehmet Z.(2006).Detection of Molluscum contagiosum Virus (MCV) Subtype I as a Single Dominant Virus Subtype in Molluscum Lesions from a Turkish Population. Archives of Medical Research 37) 388–391.

[8] Sam brook J.,Fristsch ,E.F, and Maniatis T. .(1989) Molecular Cloning : A laboratory Manuale,2ed Cold spring harbor press ,cold.

[9] Monica Agromayor, Mariano Esteban, Pablo Ortiz, Jose L. Lopez-Estebaranz, and Antonia Martin-Gallardo, Josefa Gonzalez-Nicolas,(2002).Molecular Epidemiology of molluscum Contagiosum Virus and Analysis of the Host-Serum Antibody response in Spanish HIV-Negative Patients. Journal of Medical Virology 66:151±158 .

[10] Porter CD, Archard LC. (1992): Characterization by restriction mapping of three subtypes of molluscum contagiosum virus. Journal of Medical Virology 38:1–6.

[11] Carol H. Thompson.(1997). Identification and typing of molluscum contagiosum virus in clinical specimens by polymerase chain reactionJournal of Medical Virology 53:205–211 .

[12] Chandrashekar L, Devinder M, and Telanseri J,(2002). Clinical profile of molluscum contagiosum in children versus adults Dermatology online Journal 9 (5): 1

[13] Dohil MA, Lin P, Lee J, Lucky AW, Paller AS, Eichenfield LF. (2006).The epidemiology of molluscum contagiosum in children. Journal of Amirecan Academy Dermatol. Jan;54(1):47-54.

[14] Kuchabal, D.S. Kuchabal, B. Siddaramappa, P.S.M. Katti & P.V. Patil(2011). Molluscum Contagiosum a clinical and epidemiological study. Internet Journal Of Dermatology. Volume 8 Number 2.

[15] Zandi F, Shamsaddini S, Kambin N,(1999). Prevalence of Molluscum Contagiosum in students of elementary schools of Kerman. Iranian Journal of Dermatology.Vol. 2,No. 7.

[16] Mostafa FF , Hassan A, Soliman M I, Nassar A, Deabes R H (2012). Prevalence of skin diseases among infants and children in Al Sharqia Governorate, Egypt. Egyptian Dermatology Online Journal ,Vol. 8 No 1:

[17] Talia Kakourou MD, Asimina Zachariades. (2005). Molluscum contagiosum in Greek children: a case series nternational Journal of Dermatology;Volume 44, Issue 3, pages 221–223**.**

[18] Samir S. S, (2009) Denouement and discussion: Molluscum contagiosum .Arch Pediatr Adolesc Med.;163(4):384-384.

[19] Yamashita H, Uemura T, Kawashima M. (1996).Molecular Epidemiologic Analysis Of Japanese Patients With Molluscum Contagiosum. Int J Dermatol;35:99-105.

[20] Sara SebnemKilic and FatihKilicbay (2006) Interferon -ɤTreatment of MolluscumContagiosum in a Patient With Hyperimmunoglobulin E Syndrome. journal of the American Academy of Pediatrics, Volume 117, Number 6.

[21] Scholz J, Rosen-Wolff A, Bugert J, et al. Epidemiology of molluscum contagiosum using genetic analysis of the viral DNA. *J Med Virol*. Feb 1989;27(2):87-90

[22] Jean L, Residence V.(2004).Treatment of molluscum contagiosum with 585 nm collagen remodeling pulsed dye laser .. European Journal of Dermatology. Volume 14, Number 2, 103-6.

[23] Magdalene AD, Peggy L ,Amy S, James L, Lawrence F.(2006). The epidemiology of molluscum contagiosum in children .Journal American Academy for Dermatology .;54:47-54.

[24] Clark Stevens .(2009). Molluscum infection information.azian article. Cohen J, Powderly WG. (2004). Infectious Diseases. 2nd ed. New York, NY: Elsevier;:2053-2056.