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Hepatitis C Virus Genotypes isolate using linear array the Roche method: Comparison of that Genotypes isolate with early researched source from Middle East, North and Central Africa, Mediterranean and other regions of world

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ABSTRACT

Precise determination of HCV genotypes could provide guideline for finding the pathological conditions of specific genotypes of infection in liver and decision making in antiviral treatment, because of each genotype having different antiviral drugs responsibility. Isolation of specific types of HCV genotypes infection in a specific region of country has an important role on community health management and police making, since each genotype having different pathogenicity and distribution. The present study was to isolate specific HCV genotypes among Sirt population in Libya and compared that with an early scientific researched data from Arab and non-Arab countries. A total of seventy three males and forty two females anti HCV positive patients were received from serology division after primary screening, confirmed with PCR conventional method and further genotyped by using Linear Array the Roche method, during the period of 2011 to 2013. In present study, 63.5% were males and 36.5% were females' hepatitis C virus infected cases. Among that, 67.82% of genotype 4 was isolated and most circulating type in Sirt region of Libya followed by genotype 1, 2 and 3 with clinical history of acute and chronic condition (18.30%, 7.82%, & 6.1%). Genotype 5 and 6 were not isolated in this population. The current study highlighted that genotype 4 is common circulating type after genotype 1 and both types are accounted about 85.5 percent of HCV infection in Sirt city and surrounding villages. Genotypes 4 and 1 have more severity of liver diseases as compared to the genotype 2 and 3 confirmed by viral load analysis. The genotypes of this isolated result have been compared with an early published data from Middle East, Mediterranean regions; North Africa, Central Africa and other regions of the World for recognize the reasons, highest and least infectivity of Hepatitis C virus genotype 4 with subtypes. An early researched data was obtained from open and free access journals by Google engine search.

Key words: Liver Cancer, HCV genotypes, RT-PCR, Linear Array

Abbreviation: Real Time Polymerase Chain Reaction

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1. INTRODUCTION

Hepatitis C virus is an important agent of chronic hepatitis, cirrhosis and hepatocellular carcinoma. It has been observed maximum genetics diversity in its nucleotide sequence. So far six main types of genetic heterogeneity with 90 subtypes have been isolated (Robertson et al.1998). HCV genotype 4 is an extremely assorted genotype confirms major divergence and an extra subtypes (4a,4c,4d,4e,4f,4g,4h,4k,4l,4m,4n,4o,4p,4q,4r,4s,4t,4u) compared with other genotypes (Rapicetta et al.1998; Simmonds et al.2005) and need to be focused much in clinical practice. The genetic heterogeneity of HCV has been typed into four hierarchical branches; genotypes, subtypes, segregate, and mutant-species (Simmonds et al.1995). HCV genotypes assay has emerged as very important tool in predicting a sustained response and in confirmation the duration of antiviral treatment. There are different methods are available in the molecular diagnostic laboratory likes direct DNA sequencing (Bukh et al. 1992; Germer et al.2003), type specific PCR (Okamoto et al. 1992), restriction fragment length of polymorphisms; line probe assays (Nakao et al. 1991), primer specific and mispair extension analysis (Hu et al. 2000), heteroduplex mobility analysis by temperature gradient capillary electrophoresis (Margraf et al. 2004) and denaturing high performance liquid chromatography (Liew et al. 2004) are manual and semi automated study. Beside this methods, also others kits methods are available in routine clinical laboratory likes Inno-liba HCV linear array (Belgium), Roche Linear array (Roche), Trugene HCV RNA 5NC genotyping kit (Bayer) and Versant HCV genotypes assays. However, these methods were not finds to be accurate in subtype isolation. The HCV RNA sequencing and phylo-genetics analysis are the best and accurate in isolation of all genotypes and subtypes at all region of the world (Pawlotsky et al. 2002). The previous study was in capital city of Tripoli with limited data and the present study was in Sirt city to isolate genotypes by using the method of linear array. This assay is quick and best to isolate HCV genotypes in clinical diagnostic laboratory but not for subtypes. HCV genotype 4 is most predominates in Arab heritage with same mothers tongue. Hence, current study have compared with existing data from Arab and non-Arab heritage countries within Middle East, North Africa, and Central Africa, South Europe in Mediterranean and others countries in the world, for finding the major and minor circulation of HCV genotypes 4 and others. This study might be key role in finding the epidemiological factors as well preventing the infection rate and in guiding antiviral treatment as well as vaccine preparation for this country in aspect.

Liver Cancer: Liver cells damage by hepatitis C virus.

RT-PCR: Real Time Polymerase Chain Reaction technology for gene amplification.

Genotype: Alteration in genetics material against environment to create several subtypes.

Antiviral drugs: PEG-Alfa interferon 2b and Ribavirin for suppression of hepatitis C virus replication.

2. SCOPE OF STUDY

The scope of study is to isolate HCV genotypes for antiviral treatment decision making and comparatively analyzed for epidemiological aspect. This study might be helpful to complete clearance of viral load from blood circulation to prevent hepatocellular carcinoma among population in feature.

2.1. Materials

Patient's sera were obtained from one hundred fifteen Libyan patients who were Anti-HCV positive by primary screening. There were one fifteen selected patients for HCV genotyping include males and females. Patient's ages ranged between 20-80 years of age but higher number of infectivity between 25 to 40 years age groups. All samples were well documented and verified for HCV positivity and then stored at -80^{o} C. After that the sample was utilized for PCR amplification and genotyping through Cobas Amplicor and Linear Array the Roche model. HCV RNA was extracted from serum by using chemotropic agent procedure with adaptation to the manufacture guideline (Roche Cobas Amplicor, USA).

2.2. Methods

2.2.1. Cobas Amplicor PCR

The Cobas Amplicor HCV test, version 2.0 uses the primers KY78 and KY80 (Roche molecular system, USA) to define a sequence of 244 nucleotides within the highly conserved 5-untranlated region of HCV genome with maximum sequence conservation among different types of HCV genotypes. For PCR amplification, 50ul of diluted HCV RNA and 50ul of master mix was used for the reverse transcription and amplification reaction in same reaction mixture with thermo-stable recombinant enzyme *Thermus thermophilus* DNA polymerase. Following reverse transcription of the HCV target RNA and the control RNA, the reaction mixture is heated to denature the RNA: cDNA hybrid and expose the primer target sequences. As the mixture cools, the upstream primer (KY80) anneal specifically to the DNA strand, rTth pool extends the primer, and a second DNA stand is synthesized. These complete the first cycle of PCR, yielding a double DNA and expose the primer target sequences.

Comparison: HCV Genotype 4 is major predominant in Arab heritage than other non Arab custom. Also, it is least circulating type in where the population disturbance and tourist visitor. Since a decade this genotype has been least spreader to some part of world where it is not predominate like USA, Canada, and South India. This might be permanent immigration between the regions and sign of country developing as well infecting virus.
Content: The information which is briefly mentioned in this project work was obtained from research articles.

2.2.2. HCV RNA Quantification

After amplification reaction complete, the Cobas Amplicor analyzer was automatically added

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Table 1

HCV Genotypes frequency in Sirt province

HCV Genotypes	%	Frequency
Genotype-1	18.30	21
Genotype-2	7.8	9
Genotype-3	6.1	7
Genotype-4	67.82	78

Table 2

HCV GT4&others distribution in Middle East (Arab, non Arab countries), Africa (NA, CA, WA, EA&SA) Mediterranean (S. Europe) and other part of World

1.Arab Countries	**Genotypes/Subtypes Most/Less common**		**Arab Countries(1)**	**Genotypes/Subtypes Most/Less common**	
Saudi Arabia	4	1a,1b,3a	Kuwait	4	1
Yemen	4	-	Iraq	4	1a,1b,3a
Oman	4	1a,1b	Syria	4	1b,1a,3a,5
Bahrain	4	1a,1b	Lebanon	4	1a,1b,2,3
Qatar	4	2,3,	Gaza Strip	4	1,3a
UAE	4	**3a**	**Jordan**	**1a**	1b**,4**
2.Non-Arab Countries			**African Countries-continue(3)**		
Iran	1a,3a	1b,**4**	**C.A**.Republic	4	4k
Israel	1b	1a,2	Congo	4	4c,4k,4r,4a
Turkey	1b	1a	Liberia	4	---
3.African Countries			Uganda	4	**4r**
Libya-Sirt (N.A)	4	1,2,3,	Tanzania	4	-
Egypt	4a	1a,1b,2as	Rwanda	4k	-
Gabon	4e/c	1a,1b,2a,2b	**Tunisia**	1b	1a,2a,2b,3a**,4a**
Nigeria	1,4	2	**Morocco**	1b	2a,2c,1a,**4**
Sudan-(**E.A)**	4	4e,4c,4d	**Algeria**	1b	2,**4**,3,5
Cameroon	4	1,2	Mauretania	-	NA
Ghana(**W.A)**	2b	--	S. Africa**(S.A**)	**5**	1,2,3,**4**
4.South Europe(Mediterranean Region)			**Other Parts of World**		
Italy	1b	1a,2b,**4,4d**	S. India	3a,1a,1b,2a	**4a**
France	1b	1a,2,3a,**4a,4d**,5a	USA	1b,1a	2b,3a,**4**,5
Spain	**4c/d**	**4a,4e,4**	Europe	1a,1b	2,3a**,4,**
Greece	1b	**4a**	China/Japan	1b,	2a,2b,3a,3b

Notes: NA-North Africa-EA-East Africa-CA-Central Africa-WA-West Africa-SA-South Africa

100µl denaturation solution to the amplification tubes to chemically denature test target and quantitative standard amplicons to form single-stranded DNA and hybridized to the biotin-labeled amplicon. Following hybridization reaction complete the Cobas analyzer washed the magnetic beads and added avidin-horseradish peroxidase conjugate, and upon addition of TMB, a color complex, the absorbance (*A*) of which was measured at 660 nm, was produced as results.

2.3. Linear Array HCV Genotyping-Roche

After denaturation of HCVRNA PCR amplification and detection steps complete, 100µl of denatured PCR product (amplicon) are then added to the appropriate well of the typing tray that contains 4ml working hybridization buffer and designed Linear arrays HCV genotyping strips, which are coated with sequence of oligonucleotide probe specific for various HCV genotypes (Roche molecular system, USA). The biotin-labelled HCV amplicon will hybridize to the genotype-specific oligonucleotide probe only if the amplicon contains the matching sequence of the genotype-specific probe. The Streptavidin-Horseradish Peroxidase Conjugate (Roche molecular system, USA) binds to the biotin-labelled amplicon hybridized to the genotype specific oligonucleotide probe on the strip. The strip is washed several time by distilled water to remove any unbound Streptavidin-Horseradish Peroxidase Conjugate and then a substrate solution containing hydrogen peroxide and 3, 3, 5,5-trtramethylbenzidine (TMB) is added to each strip. Blue colour formed after the catalyses the oxidation of TMB in which precipitates at the probe positions where hybridization occur. The Linear Arrays HCV Genotyping Strip has then read visually by comparing the reference pattern of positive (blue) and faint bands of genotype patterns.

3. RESULTS

Among one hundred fifteen patients studied, 63.5% were males and 36.5% were females infected population. The HCV chronic infection was more in 28.70% female patients aged (31-50 years) and 51.30% male patients aged as 31-

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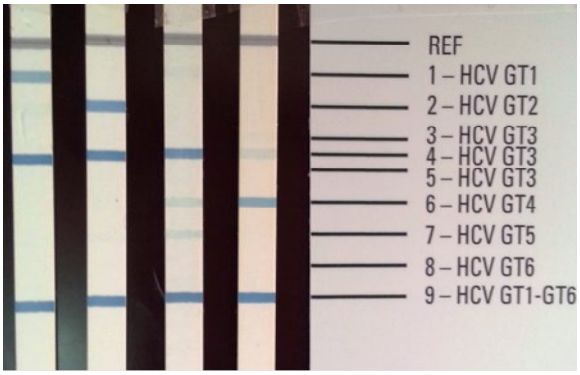

Figure 1
HCV Genotypes isolate

50 years. Besides that, according to the age and sex groups, the patient sample was taken for PCR and genotypes analysis. The age and sex group was from 20 to 80 years, but out of one hundred fifteen patients, the 21 to 30 years age and sex group was 10 females and 20 males respectively. from 31 to 40 years was 12 females and 27 males from 41 to 50 years was 11 females and 12 males from 51 to 60 years was 4 females and 5 males and from 61 to above years was 4 females and 7 males. However, the age groups and sex of this HCV acute and chronic infection was between the age of 31-50 years of female and male population. In the studies of HCV genotypes analysis; we found the genotype4 67.82% was most prevalence and circulating subtype in Sirt region of Libyan population followed by genotype 1 18.30%, genotype2 7.8% and genotype3 6.1%.Genotype1 has second most circulating type in this region after genotypes 4 (Table 1). In this study, there were 55.65% of GT4 patients had severed chronic hepatitis and 12.17% of patients were confirmed as acute hepatitis according to the viral load in general population. Genotypes GT1, GT2 patients and GT3 patients were detected and there were chronic and acute cases also (Figure 1). The male populations are higher than female populations in the percentage of HCV infectivity. The most affected population between the age groups of male and female were 25-50 years and more viral load has been observed. The high viral quantification was observed in genotypes 4 and genotype 1 in general HCV infected population. The maximum viral quantification was 7 log10. The genotypes 5 and 6 were not detected in this study. These isolated results have compared with early published data in where genotype 4 more or least predominates and clearly briefed in discussion (Table 2).

4. DISCUSSION

Hepatitis C virus genotype 4 is the most predominant with different subtypes circulating in the Middle East (Sanaa et al. 2008) and Africa (Libya, Egypt and Central Africa) (Daw et al.2002; Xu et al.1994). Also, second least circulating genotype in Sothern Europe (Mediterranean regions) (Argentini et al. 2000; Sanchez et al. 1997), Non Arab Country in Middle East (Shemer et al. 1998; Sawant et al. 1998; Bozdayi et al. 2004), South India (Raghuraman et al. 2004), and USA (Lyra et al. 2004) through immigrant, travelers and others routes of transmission followed by genotype 1,2 and 3. Egypt has highest prevalence (15%) of HCV with approximately 90% infections caused by genotype 4 (Egyptian Ministry of Health. 2007). The scientific research on genotype 4 and others have not been studies adequately. Therefore, in feature, need to concentrate on 20% Middle East and the Arab country among 170million population in all over the world (Sanaa et al. 2008; Argentini et al. 2000; Shemer et al. 1998; Swant et al. 1998; Bozdayi et al. 2004).

Present study was isolated as most predominant genotype 4 in Sirt region after 1, 2, and 3 types (Table 1). This study has been absolutely correlated with earlier studies in Tripoli (Daw et al. 2002) and in all Arab Nations (Shobokshi et al. 2003; Dhahry et al. 1993; Alfaresi et al. 2011; Pacsa et al. 2001; Chironna et al. 2003; Antaki et al. 2010; Mahfoud et al. 2010; Shemer et al. 1998; Bdour et al. 2002), Mediterranean region and Africa. Fascinatingly, there are four model for HCV genotypes distribution have been tabulated with early published data, especially genotype 4: one is most frequent for Arab Nations, where genotypes 4 predominates excluding Jordan, while the second one is specific for non-Arab countries likes Turkey (Bozdayi et al. 2002), Israel (Weinstein et al. 2001) and Iran (Sawant et al. 1998), where genotype 1 predominates (Non-Arab countries).Third one is genotypes 4 were more circulating types in Central, and North Africa (Libya and Egypt) ,whereas the Arab and non Arab tradition. As well as in Jordan1a (Bdour et al. 2002) is the main subtype (40%) after that 1b (33.3%) and then genotype 4(26.4%) is least frequent than other Arab countries, because of it might be restricted migration or other reason. The fourth one is least circulating genotypes 4 in Southern European countries within Mediterranean regions have been recently reported except Spain. Also in UAE, genotype 3 second most dominant followed by genotypes 4 because of permanent residence and business between India and UAE since petrol discovery, but comparing with others Arab Nation it is second most circulating type. In same time, genotype 4 is very common in Gaza Strip then in Israel which is neighborhood country and 1b, and 1a is common subtypes. Due to immigration between Gaza (Shemer et al. 1998) and Egypt, the genotype 4 has been circulated since so many years but it is not in Israel because of restricted for migration and other activities.

Genotypes 1, 2, and 3 are very frequent genotypes globally. Subtype 1a is the most frequent after 2b and 3a in North America and Northern Europe (Mahaney et al. 1994; McOmish et al. 1993). Subtype 1b is most common in Southern and Eastern Europe after genotype 2 and 3 (Nousbaum et al. 1995; Viazov et al. 1997). Although HCV subtype 2a and 2b are relatively common in North America and Europe, subtype 2c is found mainly in Northern Italy (Osella et al. 1999). Genotype 3a, which is prevalent in South East Asia (McOmish et al. 1994), it has been circulated by immigration and travelers in Europe and the USA and with comparatively high incidence in intravenous drug

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abusers (IVDA) (Silini et al.1995; Pol et al. 1995). Genotype 5 has been isolated in South Africa followed by 1,2,3, and 4 (Smuts et al. 1995). Genotype 6 has been isolated in Hong Kong, Vietnam and all over the South East Asia (Simmonds et al. 1996; Mellor et al. 1996; Sugiyama et al. 1995). In agreement with present study, there was no genotype 4 isolated in most part of Asia (except South India), Northern region of Europe and South America.

Arabian North African countries such as, Egypt, Libya, Tunis, Algeria, Morocco and Mauretania are considered as Maghreb region. The HCV genotype 4 is most predominates followed by 1a 1b and 2a in Egypt (Ahmed et al. 2011; Esmat et al. 2011) also it is more or less common patterns in Libya (Daw et al. 2002) like Egypt it seems that immigration between two countries since so many years. These predominates genotypes 4 and others patterns were totally different from others parts of Arabian custom of North African countries likes Tunisia (Ayed et al. 2003), Algeria (Samir et al. 2013), Morocco (Sekkat et al. 2008) and Mauretania because of French colonization since so many years. The genotypes 1b is more circulating type after 2a, 2b, 2c, 3, and least 4. These patterns are more similar to French (Nicot et al. 2005) and Sothern European countries. Beside this patterns, in Central African countries likes Gabon(Xu et al.1994), Nigeria (Oni et al. 1996), Sudan (Gasim et al. 2012), Cameroon (Ndjomou et al. 2003), Central African Republic, Congo, Liberia, Uganda, Tanzania and Rwanda (Simmonds et al. 2004; Smith et al. 1997), the genotype 4 and subtypes are very common and more or less similar like Middle East (Arab), Libya and Egypt but the subtypes might be different within the genotypes 4.It might be circulated through immigration, population distribution and others factors between Arab and non-Arab custom. Genotype 2b is predominates in West African county of Ghana (Daniel et al. 2003) after others types. Genotype 5 is circulating type in South Africa (Smuts et al. 1995) after 1, 2, 3, and least genotype 4.

Mediterranean region in Southern Europe countries likes Italy (Ansaldi et al. 2005), France (Nicot et al. 2005), and Greece (Katsoulidou et al. 2006) having least circulating genotypes 4 than Spain (Fernandez et al. 2006) where genotype 4 more predominate. Also, since a decade genotype 4 has become infected many patients in Europe (Argentini et al. 2000), USA (Lyra et al. 2004) and Southern India (Raghuraman et al. 2004) due to immigration (Table 2). The HCV genotyping as an epidemiological marker has been noticeably authenticated, generally in suggesting the source of infection and make clear the possible mode of transmission in different region of world. Even though, the issue of pathogenicity of the different genotypes/subtypes is still incomplete discovery. Further, the complete scientific analysis is still needed to create original clinical research data on HCV genotypes in different groups of populations.

5. CONCLUSION

Libya is an Islamist state in North Africa with Arabian heritage. Now, It is really understand that HCV genotypes 4 is most circulating type in Libya, Egypt, Middle East, Central Africa and least circulating type in Southern Europe of Mediterranean regions and where the permanent residence and travelers visit between the countries. Hence, need to provide an extra awareness to this hepatitis C virus genotype4 and subtypes infection, which is leading to cirrhosis, hepatocellular carcinoma, liver failure and finally liver transplantation. Also, Country economic growth might be slow down because of an illness and death by this killer virus. So prevention of HCV infected population through antiviral treatment is necessary and an instant action to be taken in each country including Libya.

SUMMARY OF RESEARCH

1.Patients sample received from serology after primary screening and confirmed with the conventional method of PCR analysis.
2.HCV genotypes isolated by using method of linear array after PCR amplification. This analysis has major role in antiviral treatment and decision making.
3.This report compared with existing data from Arab and Non Arab heritage for epidemiological aspects.

FUTURE ISSUES

This isolate is more useful for anti viral treatment direction in this locality and police making.

DISCLOSURE STATEMENT

The authors declare that they have no conflict of interests.

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