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Frequency of Hepatitis C virus genotypes circulating in Gujrat Punjab, Pakistan

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ABSTRACT

Hepatitis C virus (HCV) infection is primary cause of liver disease that leads toward liver cirrhosis and hepatocellular carcinoma. This study designed to diagnose the prevalence of HCV genotypes circulating in Gujrat, Punjab. Blood samples of 3540 suspected patient were collected from July 2017 to January 2019 and analyzed for HCV infection by using qualitative PCR, confirmed cases were subjected to molecular genotyping on Real Time PCR (Applied Biosystems, Step One software). Results show 2750 out of 3540 (77.6%) suspected patients were positive for HCV-RNA. Female have higher percentage (57.78%) than male (42.22%) with highest viral load observed at age group 15 to 30 years. The genotype 3a (83.33%) was dominant to other circulating genotypes as 2b (13.33%) and mixed 3a/2b (3.33%). The highest prevalence of HCV infection was noted in females compared to males and 3a was most dominant genotype. This study highlights the new reported mixed genotypes 3a/2b in this region that should be remarkable for treatment of high resistant cases of HCV. Further studies are needed to find out the exact cause of high viral load in this region.

Keywords: HCV, ICT, ELISA, PCR

INTRODUCTION

Hepatitis C virus (HCV) infection has affected millions of people throughout the world (Webster DP *et al*., 2015). There is significant burden of HCV infection around the globe and it varies geographically. HCV infection is leading cause of various types of cancer especially hepatocellular carcinoma (Allison, 2015; Mahale, 2017). Approximately, 170,000 new cancer cases were entered into the data of GLOBOCAN 2012 (Plummer, 2016). The cancer patients with chronic HCV infection cause extra morbidity and mortality that can interfere with treatment of cancer (Mahale *et al.,* 2012; Torres *et al.,* 2015; Torres *et al.,* 2015; Mahale *et al.,* 2015; Mallet *et al.,* 2016; Economides *et al.,* 2016). The major route of HCV transmission was the use of HCV infected materials. In developing countries, the mutual use of contaminated needles during medical treatments was epidemic in 1940s. The Intravenous drug use is the major cause of HCV epidemic throughout the world (Degenhardt et *al.,* 2016; Zibbell *et al.,* 2015). Many iatrogenic factors are contributing to highest epidemic of HCV infection in under developing countries. A survey conducted by different organizations of the World, the epidemic rate of HCV antibodies ranges from 1.6% to 2.8% (Mohd *et al.,* 2013). Prevalence of HCV infection in under developed countries including the Pakistan (4.7%), Egypt (15%), Taiwan (4.4%), Australia (1.7%), North America (1.1% to 1.3%), and Europe (0.5%-4.5%). Recent reports on epidemic of HCV infection that more than 80 million of positive cases are reported worldwide (Gower *et al.,* 2014; Stanaway *et al.,* 2016). The primary cause of HCV transmission are contaminated surgical instruments, infected blood transfusion, non-sterile dental instruments, manifestation of illegal drugs, sharing of goods that are contaminated with HCV, whereas interfamilial transmission and sexual transmission have also been reported (Qureshi *et al.,* 2013). The symptoms of HCV infection may not appears for many years and about 20% of cases suffered from fatal liver injury that ultimately lead to liver cancer (Imran *et al.,* 2013). Rather there is high need to study the relationship of HCV genotype to age, sex, site of entry and chronic situation of infection in developing countries where no proper data information is available until now.

Author's Contribution: K.S., N.Z. & M.N.R, conceived and designed study plan; **M.N.R**., collected samples and did research work; **K.S. & N.Z**., analyzed data and performed interpretation; **K.S. & M.N.R.,** Did article drafted, revised and prepared for publication

This is the first study in this region that was designed to find out the prevalence of HCV infection and genotypic distribution in Gujrat, Punjab, Pakistan. There was no proper study conducted to highlight this issue previously, although large numbers of new cases were reported every day in healthcare units of the region. This study analyzed the HCV genotypes and subtypes along with the clinical and demographic parameters that describe the high risk factors associated with high prevalence of HCV infection. This research was held to expect that it will draw the attention of national and international organizations to perform their best efforts to solve this outstanding problem in low literate population. Gujrat city has insufficient availability of health facilities especially in its basic health units that are directly connected with the poor villagers. There are many methods for detection of HCV but Real Time PCR is highly sensitive and specific method among all the available methods. This system detects viral load and genotyping simultaneously of said sample. So, there are less chances of cross contaminations during this method. All molecular parameters like qualitative, quantification and genotype were performed on real time PCR because there is no real time PCR based studies on this issue in Pakistan. The available literature for genotyping of HCV from Pakistan indicates that 3a genotype is more prevalent in rural area due to use of unsafe needles and nonprofessional practitioners. There are many studies on prevalence of HCV genotypes in different cities and districts in Pakistan (Safi *et al.,* 2012; Khan *et al.,* 2014), but no such study was conducted in Gujrat, Pakistan. This study aimed to find out the frequency of HCV infection along with genotypes and subtypes in district Gujrat, Punjab, Pakistan.

MATERIALS AND METHODS

Sample collection

A total number of 3540 blood samples of Hepatitis suspected cases were collected during July 2017 to January 2019, from different hospitals of Punjab. All written data including the age, sex, district and complete address was taken from each patient.

HCV RNA qualitative and quantitative PCRs

The RNA extraction of all samples was done by using the standard method mentioned by Qaigen RNA mini extraction kit. The 50 µL of RNA was eluted as final product during the extraction procedure for further analysis. Total reaction volume of 50 µL was prepared by using the 25 µL of reaction mix and 25 µL extracted RNA. Step One Real Time PCR (Applied Biosystems) was used for qualitative and quantitative analysis using the kit method (Roboscreen, Germany). A complete set of negative control (NC), weak positive and positive control were applied with the qualitative analysis and 6 standards sets that range from higher value to lower value along with internal control (IC) was used for the quantitative analysis. This system does not import standard curve and was highly sensitive for research work. The JOE was used as target dye for HCV and FAM for internal control. The lower detection limit with this kit was 25IU/mL and precoated wells decrease the chance of false positive that result due to diluted internal control and standards. Step one Real Time PCR capable of cDNA synthesis and complete amplification procedure in the same tube. Samples with high concentration of HCV RNA were diluted 100 fold and resulted concentration was multiplied with the dilution factor and actual concentration was obtained.

HCV genotyping

The positive controls with known genotypes were used for genotypic analysis of all these samples on Step One Real Time PCR. The cDNA was synthesized by using kit method (Amplisence, Russian) in which final 25 µL cDNA was obtained that was dissolved in 25 µL buffer before storage. Equal volume of synthesized cDNA was mixed in final reaction mixture for genotype identification and two dyes were applied for allelic discrimination of analysis. Each set of primer was labeled with the specific dye for each well. Mix-A contained the primers set for 1 and 3 subtypes and mix-B with the genotype primers set that include the 2,3b, 5 and 6 subtypes.

Statistical analysis

The Minitab 17 software was used for the analysis of all data. The tests including the T-test, Chi Square and Likelihood Ration test were used to check the association in the categorical variables. The level of significance was set 0.05 and p-value less than 0.05 was set as standard for statistical significance.

RESULTS

There were 2750 positive samples out of 3540 during the initial screening and all were confirmed by using qualitative PCR. The results of the study show very high ratio (77.6%) of HCV positive cases in this region (Fig.1).

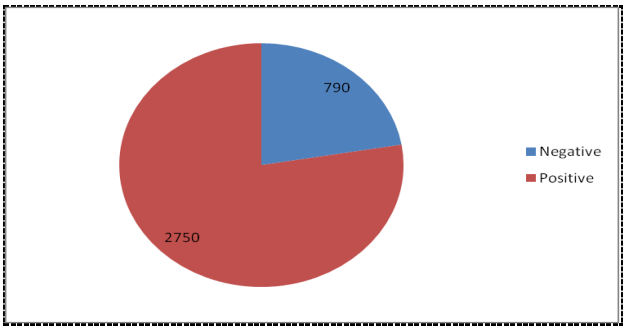

Fig. 1: Prevalence rate of HCV

Gender wise distribution of HCV positive cases clearly demonstrates that the female are at high risk (57.78%) as compared to male (42.22%) as shown in Fig. 2. All these samples were initially checked by screening method and then subjected to ELISA procedure. Some false positive results were also found at this stage that were excluded from the study and only high titer positive samples were selected for further analysis that showed the value range 2.0-3.9 on ELISA. But final confirmation was made by using qualitative PCR and only PCR positive cases were used for this study.

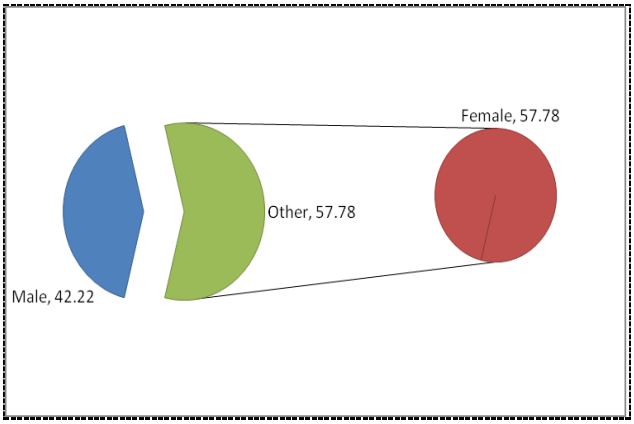

Fig. 2: Prevalence of Hepatitis C Virus according to gender

The frequency of distribution of HCV infection is different according to age and sex of patients. Low HCV prevalence percentage was noted in age group <15 years as compared to other age groups. While age group 15 to 30 years was main target for HCV RNA as compared to other age groups and high prevalence rate was seen in this age group as shown in Fig.3. The percentage prevalence of this group is high as compared to other age groups and female are more frequently affected by this infection at young age. Overall frequency of HCV prevalence was high in females as compare to male while significant difference was noted according to gender and age groups during this study.

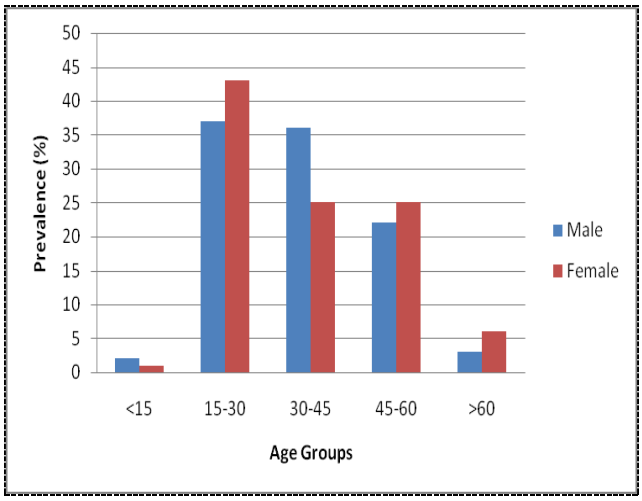

Fig. 3: Prevalence of HCV in different age groups

The genotype 3a is most dominant genotype accounting for 83.33% as compared to other circulating genotypes as 2b that account for 13.33% and mixed genotypes 3a/2b that account for 3.33%. The summary of HCV genotypes according to various age groups is provided in the Table I. The cross tabulation and chi square method was used to find the percentage among the individual genotypes. On other hand, the prevalence of the each genotype among one age group can easily be cross checked with same genotype in other age group. Now it can be observed from table I that most prevalent genotype is 3a. Actual percentage, prevalence frequency within the genotypes and gender is mentioned in each column. The genotype “3a” is most prevalent genotype according to gender and genotypes. During this study date was statistically significant because difference was observed comparing the male and female for each type of genotype as individual p values for each group were noted <0.05. Genotype “2b” is second most prevalent genotype in this region as no study was done in this region on HCV genotype and mixed genotypes are present in small percentage as shown in table I.

Table I: Summary of HCV genotypes according to various age groups.

Gender	Genotype							Total
	1a	1b	2a	2b	3a	3b	Mixed	
Female% Within Gender% Within Genotype	0	0	0	104	647	0	26	777
	0%	0 %	0%	13. 33 %	83.33 %	0%	3.33 %	100%
	0%	0 %	0%	55. 91 %	58.60 %	0 %	49. 05%	57. 76%
Male% Within Gender% Within Genotype	0	0	2	82	457	0	27	568
	0%	0 %	0. 352 %	14. 43 %	80.45 %	0%	4.75 %	100%
	0%	0 %	0%	44. 08 %	41.39 %	0 %	50. 94%	42.23%
Total	0	0	2	186	1104	0	53	1345

DISCUSSION

This study designed to determine the frequency of HCV and its genotypes in Gujrat, Punjab. All samples were categorized on the basis of gender and age groups. The HCV genotypes were correlated with gender and it was reported that there was variation of HCV genotypes in both sexes in this region. Previous studies in Pakistan reported that there was no difference in prevalence of HCV genotypes in male and female patients (Ali A *et al.,* 2011; Anwar I *et al.,* 2013). But our results contradicted previous reported study that there was no variation among in distribution of HCV genotypes among males and females. While in our findings most circulating genotype was 3a and it was reported by previous studies in different districts/cities of Punjab, Pakistan and it was confirmed that 3a is the predominant genotype in Pakistan (Nazir *et al.,* 2017;Khan *et al.,* 2014; Ali *et al.,* 2011; Inamullah *et al.,* 2011; Saleha *et al.,* 2014; Khan *et al.,* 2011). The results of our study are resembled with a study that was conducted in Lahore and concluded that the 3a genotype is most prevalent genotype in the study region (Ahmad *et al.,* 2010). Form another prevalence study that was conducted in Baluchistan, Pakistan reported that predominant genotype was 3a (Afridi *et al*., 2009). Our study showed that 3a is most predominant genotype in Gujrat region is in agreement to previous studies conducted in Asian population. Our results show 13.3% 2b genotype first time isolated in region. 2b genotype is already reported in Europe but ratio of genotype 2b in our study is low as compared to European papulation. While in North Africa and Middle East high prevalent genotype is 4 and in other countries like Hong Kong and South Africa 5 and 6 genotypes were reported (Waheed Y *et al.,* 2015). Other genotypes 4, 5 and 6 were not isolated in our study, showing their absence in our region (Inamullah *et al.,* 2011). Our study shows that there is no variation among the prevalence of HCV "3a" genotype in Gujrat and other geographical regions of Pakistan. Our study highlights the second more prevalent genotype "2b" in this region that was not reported earlier. The genotype "2b" may be the most important for physician and researchers to find out the exact cause of severity of HCV infection. Another important aspect of this study was the isolation of mixed genotype because their prevalence rate was interesting for finding the genotype in other region of Pakistan (Qureshi *et al.,* 2013). These mixed genotypes had enough viral loads showing that mixed genotypes were not because of low HCV levels and mixed HCV genotypes were already reported from other regions of Pakistan (Khan *et al.,* 2011). These findings suggest that the presence of new genotype "2b"and mixed genotypes are most health related problem in Pakistan that would be difficult to treat the patients. The mixed genotypes will be more interested if these are sequenced properly and are isolated in to their individual genotypes. There may be a new pattern that should prove a gateway in treatment of these patients in future. Our study indicates that the high distribution of HCV infection was observed in age group 15-30 years. These results are in accordance with previously published studies that high prevalence of HCV infection was seen in age group less than 40 years (Inamullah *et al.,* 2011; Saleha *et al.,* 2014). The genotype 3a was found most prevalent genotype in all regions of Pakistan because people were exposed to contaminated needles and non-sterilized dental equipment's. In Pakistan the trend of using needles is maximum among all under developing countries (Ahmad *et al.,* 2010). Other reported data contributed that barbers are also participating in spreading of HCV infection to healthy population due to lack of hygienic practices in barber shops and salons.

CONCLUSION

Our study concluded that 3a is most prevalent genotype among all circulating genotypes in this region. It is the first study showing that genotype 2b is the second most prevalent genotype that was not reported by any study since now. The overall prevalence rate of HCV infection in Gujrat is much high as compared to other districts and females are more frequently infected as compared to males. Another interesting finding of this study is that the rate of HCV infection is higher in age group 15-30 year as compared to other age groups which shows the need to inform the medical professionals to take important steps and start campaign so that further spreading of HCV can be controlled.

REFERENCES

Afridi, S., Naeem, M., Hussain, A., Kakar, N., Babar, M. & Ahmad, J. 2009. Prevalence of hepatitis C virus (HCV) genotypes in Balochistan. *Mol Biol Rep*. 36:1511–6.

Ahmad, W., Ijaz, B., Javed, F., Jahan, S., Shahid, I., Khan, F. & Hassan, S. 2010. HCV genotype distribution and possible transmission risks in Lahore, *Pakistan. World J Gastroenterol*. 16: 4321–8.

Ali, A., Nisar, M., Ahmad, H., Saif, N., Idrees, M. & Bajwa, M. 2011. Determination of HCV genotypes and viral loads in chronic HCV infected patients of Hazara Pakistan. *Virol J*;8:466.

Ali, S., Ali, I., Azam, S. & Ahmad, B. 2011. Frequency distribution of HCV genotypes among chronic hepatitis C patients of Khyber Pakhtunkhwa. *Virol Journal*.;8

Allison, R. D., Tong, X., Moorman, A.C., Ly, K. N., Rupp, L., Xu, F., Gordon, S. C. & Holmberg, S. D. 2015. Increased incidence of cancer and cancer related mortality among persons with chronic hepatitis C infection, 2006-2010. *J Hepatol.*; 63:822 828.

Anwar, I., Rahman, M., Hassan, U. & Iqbal, M. 2013. Prevalence of active hepatitis C virus infections among general public of Lahore, Pakistan. *Virology Journal*; 10:351.

Degenhardt, L., Charlson, F., Stanaway, J., Larney, S., Alexander, L.T., Hickman, M., Cowie, B., Hall, W.D., Strang, J., Whiteford, H. & Vos, T. 2016. Estimating the burden of disease attributable to injecting drug use as a risk factor for HIV, hepatitis C, and hepatitis B: findings from the Global Burden of Disease Study 2013. *Lancet Infect Dis*. 2016; 16:1385-1398.

Economides, M.P., Mahale, P., Turturro, F., Hosry, J., Samaniego, F., Granwehr, B. P. & Torres, H. A. 2016. Development of non-Hodgkin lymphoma as a second primary cancer in hepatitis C virus-infected patients with a different primary malignancy. *Leuk Lymphoma*. 2016. 58:485-488.

Gower, E., Estes, C., Blach, S., Razavi, S. K. & Razavi, H. 2014. Global epidemiology and genotype distribution of the hepatitis C virus infection. *J Hepatol*;61(suppl 1):S45-S57.

Imran, M., Manzoor, S., Ashraf, J., Khalid, M., Tariq, M., Khaliq, H. M. & Azam, S. 2013. Role of viral and host factors in interferon based therapy of hepatitis C virus infection. *Virol J* 10:1.

Inamullah, I. M., Ahmed, H., Ghafoor, U., Ali, M., Ali, L. & Ahmed, A. 2011. Hepatitis C virus genotypes circulating in district Swat of Khyber Pakhtoonkhaw, Pakistan. *Virol Journal*.;8:16.

Khan, N., Akmal, M., Hayat, M., Umar, M., Ullah, A., Ahmed, I., Rahim, K., Ali, S., Bahadar, S. & Saleha, S. 2014. Geographic distribution of hepatitis C virus genotypes in Pakistan. *Hepat Mon*. 14(10):1–5.

Khan, S., Attaullah, S., Ayaz, S., Khan, N., Shams, S., Ali, I., Bilal, M. & Siraj, S. 2011. Molecular epidemiology of HCV among health care workers of Khyber Pakhtunkhwa. *Virol Journal*. ;8:105.

Khan, T., Mehr, M., Ullah, H., Khan, H. & Iman, N. 2014. Frequency of hepatitis C genotypes in the north of Pakistan. *Gomal J Med Sci*; 12:9–106.

Mahale, P., Kaseb, A. O., Hassan, M. M., Torres, H. A. 2015. Hepatocellular carcinoma as a second primary cancer in patients with chronic hepatitis C virus infection. *Dig Liver Dis*; 47:348-349.

Mahale, P., Kontoyiannis, D. P., Chemaly, R. F., Jiang, Y., Hwang, J. P., Davila, M. & Torres, H. A. 2012.Acute exacerbation and reactivation of chronic hepatitis C virus infection in cancer patients. *J Hepatol*;57:1177-1185.

Mahale, P., Torres, H. A, Kramer, J. R., Hwang, L. Y., Li, R., Brown, E. L. & Engels, E. A. 2017. Hepatitis C virus infection and the risk of cancer among elderly US adults: a registry-based case-control study. *Cancer*;123:1202-1211.

Mallet, V., Van, B. F., Doerig, C., Pischke, S., Hermine, O., Locasciulli, A., Cordonnier, C., Berg, T., Moradpour, D., Wedemeyer, H. & Ljungman, P. 2016. Management of viral hepatitis in patients with haematological malignancy and in patients undergoing haemopoietic stem cell transplantation: recommendations of the 5th European Conference on Infections in Leukaemia (ECIL-5). *Lancet Infect Dis*. 2016;16:606-617.

Mohammad, H. K., Groeger, J., Flaxman, A. D. & Wiersma, S. T. 2013. Global epidemiology of hepatitis C virus infection: new estimates of age-specific antibody to HCV seroprevalence. *Hepatology*;57:1333-1342.

Nazir, N., Rasul, M. A., Asif, A., Idrees, M., Nisar, M., Zahoor, M. & Naser, M. 2017. Prevalence of hepatitis-C virus genotypes and potential transmission risks in Malakand Khyber Pakhtunkhwa, Pakistan. Virology Journal; 14:160

Plummer, M., deMartel, C., Vignat, J., Ferlay, J., Bray, F. & Franceschi, S. Global burden of cancers attributable to infections in 2012: a synthetic analysis. *Lancet Glob Health*. 2016;4: 609-616.

Qureshi, H., Mohamud, B.K., Alam, S. E., Arif, A. & Ahmed, W. 2013. Treatment of hepatitis B and C through national programme-an audit. *J Pak Med Assoc.* 63:220e4.

Safi, A., Waheed, Y., Sadat, J., lslam, S., Salahuddin, S., Saeed, U. & Ashraf, M. 2012. Molecular study of HCV detection, genotypes and their routes of transmission in north west Frontier Province, Pakistan. *Asian Pac J Trop Biomed*. 2:532–6.

Saleha, S., Kamal, A., Ullah, U., Khan, N., Mahmood, A. & Khan, S. 2014. Prevalence of hepatitis C virus genotypes in district Bannu, Khyber Pakhtunkhwa, Pakistan. *Hepat Res and Treatment*. 1–5.

Stanaway, J. D., Flaxman, A. D., Naghavi, M., Fitzmaurice, C., Vos, T., Abubakar, I., Abu-Raddad, L. J., Assadi, R., Bhala, N., Cowie, B., Forouzanfour, M. H., Groeger, J., Hanafiah, K. M., Jacobsen, K. H., James, S. L., MacLachlan, J., Malekzadeh, R., Martin, N. K., Mokdad, A. A., Mokdad, A. H., Murray, C. J. L., Plass D., Rana, S., Rein, D. B., Richardus, J. H., Sanabria, J., Saylan, M., Shahraz, S., So, S., Vlassov, V. V., Weiderpass, E, Wiersma, S. T., Younis, M., Yu, C., El, Sayed, Z, M, Cooke, G. S. 2016. The global burden of viral hepatitis from 1990 to 2013: findings from the Global Burden of Disease Study.*Lancet.*;388:1081-1088.

Torres, H. A., Chong, P. P., DeLima, M., Friedman, M. S., Giralt, S., Hammond, S. P., Kiel, P. J., Masur, H., McDonald, G. B., Wingard, J. R. & Gambarin-Gelwan, M. 2015 Hepatitis C virus infection among hematopoietic cell transplant donors and recipients: American Society for Blood and Marrow Transplantation Task Force recommendations. *Biol Blood Marrow Transplant*. ;21:1870-1882.

Torres, H. A., Mahale, P., Blechacz, B., Miller, E., Kaseb, A., Herlong, H. F., Fowler, N., Jiang, Y., Raad, I. I. & Kontoyiannis, D. P. 2015 Effect of hepatitis C virus infection in patients with cancer: addressing a neglected population. *J Natl Compr Canc Netw*;13:41-50.

Waheed, Y. 2015. Effect of interferon plus ribavirin therapy on hepatitis C virus genotype 3 patients from Pakistan: treatment response, side effects and future prospective. *Asian Pacific J of Tropical Med*; 14:85–9.

Webster, D. P., Klenerman, P., Dusheiko & G. M. 2015. Hepatitis C. *Lancet.*; 385: 1124-1135.

Zibbell, J. E., Iqbal, K., Patel, R. C., Suryaprasad, A., Sanders, K. J., Moore-Moravian, L., Serrecchia, J., Blankenship, S., Ward, J. W. & Holtzman, D. 2015. Increases in hepatitis C virus infection related to injection drug use among persons aged 30 years - Kentucky, Tennessee, Virginia, and West Virginia, 2006-2012. *MMWR Morb Mortal Wkly Rep*. 64:453-458.