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Prevalence Of Anelloviruses (TTV, TTMV And TTMDV) In Healthy Blood Donors And In Individuals Infected With HCV In Pakistan.

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Abstract

Background

Anelloviruses (TTV, TTMV and TTMDV) have been linked to the liver condition specifically associated with non-A-G hepatitis. The aim of the current investigation was to evaluate the infection rate of these viruses in Pakistan.

Methodology

A total of 1050 serum samples (550 healthy blood donors and 500 HCV-positive individuals) from Pakistani population with diverse ages were assessed for the detection of TTV, TTMV and TTMDV genomic DNA through nested PCR amplification.

Results

The incidence rates of TTV, TTMV and TTMDV were high in the investigated groups, and significantly high in HCV positive group specifically for TTV and TTMV infection. The prevalence of coinfection of any of the three viruses was more prevalent and was significantly higher for TTV/TTMV at 62.47% in HCV and 57.7% in healthy blood donors ($p<0.05$), as compared to TTMV/TTMDV in 24.47% in HCV and 25.33% in healthy blood donors, while TTV/TTMDV in both groups were present but less significant. Furthermore, there was significant association between TTV, and age groups were revealed as compared to TTMV and TTMDV as compared to previous studies in Pakistan.

Conclusion

This was the first study to evaluate the prevalence of *Anelloviruses* infection in south Asia. A significant higher prevalence rate of *Anelloviruses* infection in overall investigated groups was examined. Moreover, future investigations are mandatory to investigate and evaluate various genotypes of these viruses in the region.

Background

Anelloviruses are small, naked ssDNA viruses, that infect humans and are widely distributed (Kaczorowska & Van Der Hoek, 2020). *Anelloviruses* mostly infect mammals and newly born infants at initial stages (Kaczorowska *et al*., 2022). Three diverse genotypes of *Anelloviruses* have been reported that infect humans so called *Torque teno virus* (TTV) with genome size of 3.6-3.9 kb, which was isolated in 1997 from Japanese individuals with post transfusion hepatitis of unknown etiology (Cancela *et al*., 2016). Subsequently, another AVs *Torque teno mini virus* (TTMV) with genome of 2.8-2.9 kb was discovered in 2000 (Li *et al*., 2018). The third genus of *Anelloviruses Torque teno midi virus* (TTMDV) having genome size of 3.2 kb was discovered in 2007 that infects humans (Pan *et al*., 2018). *Anelloviruses* family comprises of 12 genera, TTV, TTMV and TTMDV belongs to *Alphatorquevirus, Betatorquevirus*, and *Gammatorquevirus*, genera correspondingly (Sabbaghian *et al*., 2024). The *Alphatorquevirus* comprises 29 species, while there is much less data regarding the phylogenetic groups of TTMDV and TTMV (Lolomadze & Rebrikov, 2020). The ORF1 sequence comparative analysis showed prominent deviation, and TTMDV whole genome analysis suggests sequence clustering (Cebriá-Mendoza *et al*., 2021). The three genotypes share identical genomic organization such as (ORF, short GC rich stretches and UTR). Furthermore, these viruses are significantly diverse at amino acids and nucleotide levels in ORF regions (Al-Qahtani *et al*., 2016).

Moreover, *Anelloviruses* infections are significantly common in general populations with approximately 90% of prevalence rate (Kyathanahalli, Snedden & Hirsch, 2021). Various Studies indicates the TTMV infection with elevated incidence rates of infection in healthy blood donors in France (Biagini *et al*., 2000), Brazil (Vasconcelos *et al*., 2002: Niel *et al*., 2001) and in Norway (Moen *et al*., 2002), as well as in individuals with hemodialysis with 95% prevalence rate (). Furthermore, these anallloviruses have been identified in the breastmilk, amniotic fluid and cord blood (Kyathanahalli *et al*., 2023). TTMV genome has also been found in the healthy women (in cervical swabs) (Shah, Wang & Hirsch, 2020), as well as women infected with cervical cancer, in saliva, feces and in PBMCs. TTMDV genome was also found in the saliva and plasma samples in healthy blood donors in France (Biagini *et al*, 2006), in health blood donors as well as in HIV and HCV infected individuals in Iran (Fatholahi &

Bouzari, 2015), and in Italy it was detected in the serum samples in healthy individuals as well as in HCV infected individuals (Focosi *et al*., 2020). TTMDV infection was also documented in Hungary in infants infected with respiratory disorders (Burián *et al*., 2011). Subsequently, TTV infection was initially detected in serum samples so called transfusion transmitted torque teno virus (TTV) (Lolomadze & Rebrikov, 2020). Recent investigations indicated that there are other routes of transmission for TTV infection such as sexual routes, parenteral, vertical transmission and others (Jarkasi *et al*., 2018). TTV infection has been found approximately in every tissue type and body fluid, the recent TTMV infection can be spread from mother to fetus as similar viral sequences were detected in mother and infants in the serum of infants, in breast milk, cord blood and in amniotic fluid (Al-Qahtani *et al*., 2016). Conclusively, coinfections by any of these genotypes (TTV, TTMV and TTMDV) in viremia of infection have also been reported and are mostly common (Bal *et al*., 2022).

The purpose of this study is to evaluate the infection rates of *Anelloviruses* and to characterize different genotypes (TTV, TTMV and TTMDV) as well as coinfection, and to evaluate any association of infection with these viruses in Pakistan.

Materials and methods

Study Setting and sample collection

This study was carried out at Molecular Virology Lab, Department of Biosciences, COMSATS University, Islamabad, Pakistan. Human blood Samples were collected from Pakistan institute of medical sciences (PIMS) hospital, red crescent Islamabad, Fatimid foundation Islamabad and the blood banks in Islamabad Pakistan.

Ethical consideration

Preceding sampling from blood donors, ethical review board of COMSATS university approval was obtained (CUI-Reg/Notif-1488/21/982). This study implicates no risk to participants, allowing waiver of all participants consent. Their rights were not affected as no personal data was collected. Blood samples from hospitals and banks were anonymous. We only collected information regarding nationality, age and sex, and all collected information was kept strictly confidential.

Blood samples collection

A total of 550 (males and females) blood samples from healthy blood donors and 500 from HCV individuals were included in our study. Blood samples were collected randomly from healthy blood donors and HCV individuals of different age groups, and genders, belonging to different geographical locations of Pakistan. Individuals visiting to Pakistan institute of medical sciences (PIMS) hospital, red crescent Islamabad, Fatimid foundation Islamabad and the blood banks for donation during the period (2021 to 2024).

Viral DNA extraction

DNA Extraction was carried out from the serum and other samples by using commercially available kits All Prep nucleic acid isolation kit (Qiagen, USA) according to instructions of the manufacturers. Briefly, 200µl of sample material was combined with 400µl of lysis solution and then was incubated for 5 minutes at 65°C. Immediately, 0.6mL of chloroform was added, followed by gentle emulsification through inversion (3-5 times) and then sample was centrifuged for 2 minutes at 10,000 rpm. Then the precipitation solution was prepared by combining 720µl of sterilized deionized water with 80µl of provided 10x concentrated Precipitation solution. The upper aqueous phase that contains the DNA was transferred to a new tube and then 800µl of precipitation solution that was freshly prepared was added in it. Then the supernatant was removed completely (not dried). DNA pellet was dissolved by gentle vertexing in 100µl of Nacl solution. The pellet was completely dissolved. After that 300µl of chilled ethanol was added to it and DNA was left to precipitate for 10 min at -20°C. The ethanol was then removed. The pellet was then washed once with 70% chilled ethanol. Finally, through gentle vertexing DNA was dissolved in 100µl of sterile deionized water. The extracted DNA was then stored at -20°C till further processing.

Detection/Genotyping of *Anelloviruses*

Primers' Selection

Universal primer NG 779/781 (sense/antisense) were used for the detection and amplification of *Anelloviruses* in general while for genotyping, other primers like NG (779/785), NG (793/791) and NG (795/796) were used for amplification of TTV, TTMV and TTMDV specifically (Ninomiya *et al*., 2008). Primers are mentioned in the table below:

Table 1: Universal Primers and type specific primers (Ninomiya *et al*., 2008).

Primer	Round	Sequence
Universal		
NG 779 (sense)	First	5'-ACAGACGAATGGCTGAGTTT-3'
NG 781 (antisense)	First	5'-CCCGAGCCCGAATTGCCCCT-3'
TTV-specific		
NG 779 (sense)	Second	5'-ACAGACGAATGGCTGAGTTT-3'
NG785 (antisense)	Second	5' -CCCCTTGACTCCGGTGTGTAA-3'
TTMDV-specific		
NG795 (sense)	Second	5'-CGACCGAGCGCAGCGAGGAG-3'
NG 796 (antisense)	Second	5' -GCCCGAATTGCCCCTAGACC-3'
TTMV-specific		
NG 793 (sense)	Second	5' -TTTACCACGCCAGACGGAGA-3'
NG791 (antisense)	Second	5' -CTCACCTCCGGCACCCGCCC-3'

Nested PCR amplification

Using Virus-specific primers nested PCR amplification of the extracted nucleic acid was carried out according to the conditions reported previous by Ninomiya *et al*., (2008), to detect genotypes of *Anelloviruses* and then visualized via gel electrophoresis. Nested PCR was carried out encompasses of two rounds of amplification; in the 1st round, universal primers were used and in the 2nd round type specific primers were used to amplify genotypes of AVs according to the conditions reported by Ninomiya *et al*., (2008) with a total reaction of 20ul prepared including 2ul of the extracted DNA. The thermocycler conditions were Initial Denaturation at 94℃ for 2 min, Denaturation at 94℃ for 30 sec, Primers annealing at 55℃ for 30 sec (35 cycles), Extension at 72℃ for 30 sec and Final Extension at 72℃ for 7 min.

Gel electrophoresis

agarose gel concentrations of 2%. Ethidium Bromide was added to the gel with a concentration of 10ug/ml to pre-stain it. Ladders of 50 or 100 bps were used depending on product size. Immediately after the gel had solidified, the products were loaded, and 60 volts were applied initially for 5 minutes. After that, voltage was raised to 90V for 35 minutes. Further gel visualization was done by UV illuminator.

Data analysis

Statistical analyses were carried out using "R version 4.4.2" under the package "dplyr". The Chi square test was used for the significance of the *Anelloviruses* in different groups**.** Significance differences were considered at statistical value $p < 0.05$ and $p < 0.001$.

Results

Study population demographics

In the current study population, a total of 1050 plasma samples were analyzed for the presence of *Anelloviruses* DNA including TTV, TTMV and TTMDV using semi nested PCR amplification from 2021 to 2024. Out of total 1050 samples, 550 were from healthy blood donors and 500 were from HCV infected individuals. Most of the samples were from healthy blood donors from Pakistani nationals. Gender and age did not significantly diverge in healthy blood donors. Blood donors in Pakistan were significantly males. With respect to age, it appears that most of the individuals were younger than 30 years. While most HCV individuals were older than 50 years as shown below in the table.

Table 2: demographics of study population (n=1050).

		Healthy (*n* = 550) *n* (%)	**HCV (*n* = 500) *n* (%)**
Gender	Male	536 (97.4)	380 (76)
	Female	14 (2.5)	120 (24)
Age (years)	<30	121 (22)	107 (21.4)
	31–40	273 (49.6)	103 (20.6)
	41–50	111 (20.1)	118 (23.6)
	>51	45 (8.1)	172 (34.4)
Age Group (Average ± SD)	<30	(23.50 ± 3.44)	(24.45 ± 2.78)
	31–40	(35.74 ± 2.94)	(35.00 ± 2.31)
	41–50	(45.57 ± 3.04)	(45.13 ± 2.40)
	>51	(63.37 ± 7.72)	(57.53 ± 3.67)

***Anelloviruses* infection rates in the different populations**

To analyze infection rate of TTV, TTMV and TTMDV, DNA from blood was isolated from total of 1050 samples representing healthy blood donor (n=550), and HCV (n=500) infected individuals inhabiting Pakistan, DNA was detected via nested PCR as detailed in the methodology. The prevalence of TTV, TTMV and TTMDV DNA in the overall population studied is shown in figure 1. The overall prevalence rate of TTV was (84.7%) significantly higher ($p < 0.001$) than TTMV (68%) and TTMDV (37.4%). Moreover, the prevalence rate of TTV was significantly ($p < 0.001$) higher than TTMV and TTMDV in both groups. Our study also found that the patient infected with HCV exhibited significantly high infection rate ($p < 0.05$) with *Anelloviruses* as compared to healthy blood donors.

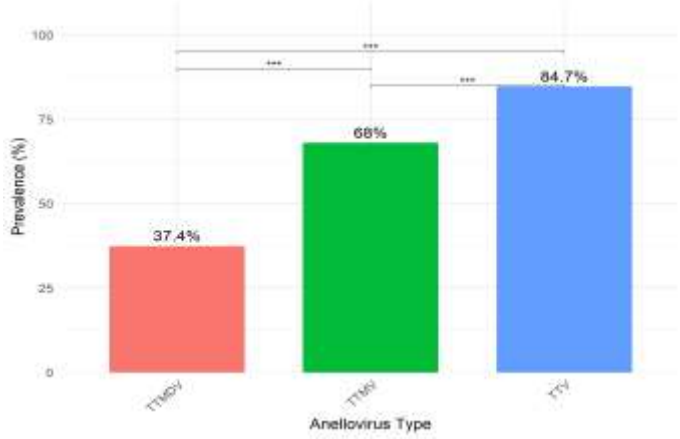

Figure 1: overall prevalence of TTV, TTMV and TTMDV infection rate in the study population (n=1050) ***significant difference ($p<0.001$).

Table 3: prevalence of *Anelloviruses* in study groups.

Virus	HCV n=500 (%)	HBD n=550 (%)	p-value
TTV	434 (86.8%)	453 (82.3%)	0.00069
TTMV	354 (70.8%)	359 (65.2%)	0.00088
TTMDV	183 (36.6%)	210 (38.1%)	0.34552
P value ***	0.00001	0.00001	

Analysis of coinfections

Our study also found that co-infections of these three *Anelloviruses* were also prevalent as shown in the Table (4). The study indicates that 62.4% of HCV infected individuals were infected significantly ($P < 0.005$) with TTV and TTMV, as well as 57.09% ($p<0.005$) of healthy blood donors and 24.4% individuals infected with TTV and TTMDV with less significance and as well TTMV and TTMDV with 8.40%. The co-infections were slightly higher in HCV individuals as compared to healthy blood donors.

Table 4: co-infections of *Anelloviruses* in both groups with significant difference ($p<0.05$).

Virus Co-infection	HCV Count	HCV (%)	HBD Count	HBD (%)	p-value
TTV & TTMV	312	62.4%	314	57.09%	0.002
TTV & TTMDV	122	24.4%	140	25.4%	0.6124
TTMV & TTMDV	42	8.40%	44	8%	0.7905

The distribution of TTV, TTMV and TTMDV infection among different age groups and gender was documented in the Table (5). There was a significant higher infection rate of TTV ($p<0.05$) among age groups and gender as compared to TTMV and TTMV infection with no significant difference in these groups.

Table 5: TTV, TTMV and TTMDV distribution in the study groups (n=1050).

		Total (n)	TTV n (%)	TTMDV n (%)	TTMV n (%)
Gender	Female	134	116 (86.50%)	48 (35.80%)	96 (71.60%)
	Male	916	772 (84.20%)	346 (37.70%)	618 (67.40%)
Age (years)	<30	228	194 (85.08%)	88 (38.50%)	151 (66.22%)
	31–40	376	310 (82.44%)	142 (37.76%)	252 (67.02%)
	41–50	229	193 (84.2%)	83 (36.2%)	160 (69.8%)
	>51	217	190 (87.5%)	79 (36.4%)	151 (69.5%)
p-value			0.0362	0.6934	0.2964

Discussion

Subsequently the discovery of *Anelloviruses*, specifically the TTV, TTMDV and TTMV have some similar characteristics like their identification in the serum of healthy blood donors and as well HCV or HBV infected individuals. The detection of these viruses in (non- A to G) infected individuals have associated them with hepatitis. This possibly validates the studies done so far, specifically on TTV, that examined the incidence of the virus in HCV or HBV infected individual's serum and in both healthy blood donors in other geographical regions. While there are limited studies on the prevalence of these viruses in the sera of healthy blood donors and in HCV or HBV infected individuals specifically in south Asia and in Pakistan.

In previous study by (Basharat *et al*., 2021) reported the prevalence of TTV in healthy blood donors and HCV or HBV infected individuals in Pakistan. However, no study regarding the prevalence of TTMV, and TTMDV were reported so far in Pakistan. Therefore, the objective of this study was to examine the prevalence of TTV, TTMV and TTMDV infection in healthy individuals as well as HCV infected individuals in Pakistan and comparison of findings to the previous studies on TTV, TTMV and TTMDV.

This study evaluated a total of 1050 serum samples from healthy blood donors (n=550), individuals with HCV (n=500). Findings from the current study indicate an overall prevalence rate of these viruses as shown in Figure (1). The prevalence rate of TTV was (84.7%) significantly elevated as compared to TTMV with (68%) and TTMDV (37.4%) correspondingly. Interesting findings were obtained from a deeper look of distribution of the three viruses in the study groups in healthy blood donors and HCV. Initially the prevalence of these viruses was significantly higher in the HCV group ($p<0.05$) in comparison with healthy blood donors. Next to that, the prevalence rates of TTV, TTMV and TTMDV were significantly different in HCV ($p<0.001$) and in healthy blood donors ($p<0.001$). Furthermore, many of the samples analyzed, irrespective of the study groups, revealed mixed infections, but the association of TTV and TTMV with ($p<0.005$) was documented suggesting being more prevalent and significantly different specifically in HCV as compared to healthy blood donors. As well as compared to other co infections like TTV/TTMDV and TTMV/TTMDV which showed less significance in both groups. The infection rate of TTV, TTMV and TTMDV were not significantly different in association with age and gender. There is diverse infection rate with respect to the prevalence of TTV, TTMV and TTMDV in different geographical regions globally. This variation can be clarified in several ways, maybe it is possibly the correct visualization of ubiquitous nature of *Anelloviruses* or may also be accredited to the probable choice of PCR amplification of target DNA and primers utilized. The high occurrence rates of *Anelloviruses* found in the viremias of this study and others can also be comparatively elucidated by how it is easy for the transmission of the virus.

The prevalence rates of TTV, TTMV and TTMDV have not much detected and reported their incidence in healthy blood donors and HCV individuals in the region. Previously study showed the prevalence of TTV with significant prevalence of 77% in healthy blood donors and similarly for HCV in India (Magu *et al* 2015). However, there is scarcity of studies on the prevalence rate of these viruses in the south Asia region. But studies in other regions reported significant prevalence rates of TTV infections (85.2%) in healthy blood donors in Qatar (Al-Qahtani *et al*., 2016), Iran (60-61%) (Salman *et al*., 2023) and China (92.5**%**) (Peng *et al* 2015).

In comparison with TTV, there is much less data available regarding the infection rates of TTMV and TTMDV in healthy blood donors and in HCV individuals in the region. Previous reports documented significant infection rates of TTMV from Qatar (60-63%) (Al-Qahtani *et al*., 2016), from France (77%) (Biagini *et al*., 2000), Norway (47-48%) (Moen, Huang & Grinde, 2002), Brazil (Vasconcelos, Cataldo & Niel (2002); Niel & Lampe (2001)) in healthy blood donors. Additionally, lower infection rates were reported in Iran (16-17%) (Ghazimorad, Bouzari & Kardi, 2014) and in Korea (40-41%) (Chung *et al*. 2004). With respect to TTMDV, the prevalence rates of TTMDV in viremia was reported significantly lower than TTV and TTMV in healthy blood donors in Italy (7-8%) (Andreoli *et al*., 2006), France (19-20%) and Korea (33-34%) (Chung *et al*., 2007). These findings show lower prevalence rate as compared to 38.27% reported in the current study in Pakistan.

Due to the lack of studies with respect to the prevalence of TTV, TTMV and TTMDV in HCV individuals, it is difficult to conclude any comparative analysis. Only one study by (Amen *et al*., 2018) reported a very low prevalence rate of TTV in HCV patients as compared to this study which shows significantly higher prevalence rate of TTV (86.93%) in HCV patients. In Qatar the prevalence of TTV has been reported 86.8% (Al-Qahtani *et al*., 2016), in Italy (79.3%) (Lapa *et al*, 2021) and in Uruguay (81%) (Cancela *et al*., 2016) in HCV individuals.

As compared to TTV, there is scarcity of data regarding the prevalence of TTMV and TTMDV in HCV patients in the region and globally. However, previous studies reported the prevalence of TTMV and TTMDV in Qatar (83% and 88%) respectively in HCV individuals. In China the prevalence of TTMV and TTMDV reported (30-40%) respectively in HCV related hepatitis (Zhang *et al*. 2023). Another study in Iran reported the prevalence of TTMV and TTMDV with 20% and 48% respectively in HCV individuals (Ghazimorad *et al*., 2014). Moreover, another study by Garcia-Alvarez *et al*. documented high prevalence of TTV and TTMV infection in HCV/HIV coinfected individuals, which is higher than 86.93% and 70.87% infection rates respectively in HCV individuals in the current study (Garcia-Alvarez *et al*., 2013). Another study also reported the prevalence rate of TTMDV with 75% and 80.5% in HIV/HCV/HBV and HIV/HCV coinfected individuals respectively in Iran (Fatholahi *et al*., 2015). Which was comparatively higher than the infection rate of TTMDV (36.53%) revealed in HCV individuals in the current study (table). High incidence of TTV, TTMV and TTMDV in HCV individuals in comparison to the low incidence of these viruses in healthy blood donors indicates similar infection routes, that are specifically compatible with these viruses like as blood and sex.

Furthermore, coinfections with multiple Anlloviruses (TTV, TTMV and TTMDV) appear to be more prevalent mechanisms (Zhang *et al*., 2023; Al-Qahtani *et al*. 2016). One of the possible explanations for the coinfections of these *Anelloviruses* analyzed in the current study and other studies would be that these viruses may have common and possibly synergetic cycles of infection that may facilitate viruses from the common family to exclusive adaptation to the environment. Another explanation would be possibly that these viruses infect the common cell types allowing the viruses to positively replicate with in the similar cells and tissues.

Conclusion

In conclusion, the current study revealed the high prevalence rate of *Anelloviruses* specifically TTV (>80%), following TTMV and TTMDV in Pakistan. Further investigations are needed to analyze and evaluate the comparative analysis of *Anelloviruses* and their genotypes in the region.

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