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Seroprevalence and Molecular Detection of Coxsackie B Virus among Patients With Diabetes Type-1 In Jigawa State

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Abstract

Type-1 diabetes (T1D) pathogenesis is either initiated or accelerated by human enteroviruses, particularly coxsackie viruses. Because the structural makeup of the virus's viral protein 2 C (P2-C) and the glutamic acid decarboxylase (GAD65) autoantigen in human beta-cells is similar, Coxsackie B viruses are one of the primary causes of type 1 diabetes. The study identified the molecular presence of the Coxsackie B virus and ascertained its seroprevalence in patients with type 1 diabetes mellitus. From March 2023 to January 2024, a hospital-based cross-sectional study was conducted in three hospitals in Jigawa State: Dutse General Hospital, Hadejia General Hospital, and Ringim General Hospital. Ninety blood samples from diabetes patients who gave their consent were collected from the research facilities. Blood sugar levels exceeding 200 mg/dL or 11.1mmol/L were used to diagnose type-1 diabetes mellitus (T1DM) in patients based on clinical characteristics. IgM indirect ELISA detection against the GAD65 autoantibody in the patients' serum was used to confirm the diagnosis of T1DM. The virus was also detected by IgM detection and molecular technique (RT-PCR). Out of 90 blood samples, 52% of all samples of T1D patients were positive for Anti-GAD65. 11 were positive for the CBV IgM antibody, indicating a prevalence of 12.2% of T1D patients across all patient groups. The RT-PCR results indicated the presence of the RNA genome of Coxsackie B virus in the serum of some T1DM patients, with a prevalence of 2.2% (2/90), as indicated by of the detection of the VP1 gene. The findings demonstrated that in Jigawa state, CBV is not the most likely causative agent of T1D; rather, risk variables for the disease include sex, age, educational attainment, parental status, and the mechanism of disease acquisition and residency. However, other factors may be taken into consideration in future research. To lower the prevalence or eradicate the disease, it may be advisable to conduct more studies on the various causative agents of T1D and to provide early treatment for affected individuals. Additionally, relevant agencies should establish public awareness campaigns on the impacts of the disease.

Keywords: Coxsackie B virus, ELISA, Glutamic acid decarboxylase65, Immunoglobulin M, Reverse Transcriptase-Polymerase Chain Reaction, Type-1 diabetes mellitus.

INTRODUCTION

Type 1 Diabetes mellitus (T1DM) has become a global burden, and at least 13 million individuals suffer from the disease worldwide (Baba *et al.*, 2012). The global annual increase in the number of T1DM patients despite the geographical variations is estimated to be 3–4% and the disease pathogenesis is either initiated or accelerated by human enteroviruses, particularly coxsackie viruses (Ahmed *et al.*, 2012). As a result of the similar structural makeup of the virus's viral protein 2 C (P2-C) and the glutamic acid decarboxylase (GAD65) autoantigen in human beta-cells is identical, Coxsackie B viruses are one of the primary causes of Type 1 diabetes and can also cause sporadic transmission in neonates in hospitals (Graves *et al.*, 2013).

Human Coxsackie B virus is a genus of tiny, single-stranded, positive-sense RNA viruses that are members of the Picornaviridae family.

The virion of Coxsackie B Virus consists of a capsid shell of 60 subunits, each of four proteins (VP1-VP4) arranged with icosahedral symmetry around a genome made up of a single strand of positive-sense RNA. According to Wild et al. (2014), Coxsackie B virus was discovered in a study comparing control and subject groups. Coxsackie B Virus infections were substantially more common in risk individuals, such as siblings of diabetes patients, when they developed anti-beta-cell autoantibodies or T1D, and in newly diagnosed diabetic patients. The idea that the virus and the disease are related was reinforced when CV-B4 was found in the pancreases of diabetes patients (Hober *et al.*, 2010).

Coxsackie B viruses are easily spread from sick individuals to healthy individuals or by direct contact with viruses expelled from the upper respiratory or gastrointestinal system. However, these infections can cause serious illness in some individuals, particularly youngsters, which can lead to lifelong issues and, in rare cases, death (Graves *et al.*, 2013).

When an uninfected person comes into contact with food or liquids tainted by the secretions of an infected person, they may indirectly contract some Coxsackieviruses. According to Berge *et al.* (2014), the viruses can endure for several days on surfaces such as door knobs and tables. The typical incubation period for the Coxsackie B virus is three to ten days. According to Atkinthon (2014), a person can spread infectious viruses even if they are symptomless, throughout the incubation phase, or after the symptoms have subsided. Symptoms of simple infections often remain for a week. The symptoms of an infected infant can include: a common cold, hypoxia, aseptic meningitis, photophobia, myopericarditis, intermittent chest discomfort, herpangina, HFMD, encephalitis, paralysis, and flaccid paralysis, which frequently affects the muscles of the proximal extremities and is asymmetrical (Akerblon *et al.*, 2012). Although infections are common throughout the year in tropical regions, the disease's peak seasons typically occur in late spring to summer. In contrast to HFMD caused by other enterovirus serotypes, notably coxsackievirus A16, patients typically have a high fever exceeding 38°C (Bahri *et al.*, 2016). Coxsackie B virus can be identified or diagnosed by either RNA detection or the production of antibodies against the virus. Research on the connection between these viruses and Type 1 Diabetes (T1D) is currently showing encouraging findings. The potential to isolate enteroviruses, mainly coxsackie B viruses, in diabetic patients' ß cells could be a clear indication of their connection (Kukreja *et al.*, 2000).

In Nigeria, the relationship between Type 1 Diabetes (T1D) and Enterovirus B was studied earlier in the serum of 40 insulin-treated Nigerian diabetic patients, using complement fixation. A higher level of anti-Coxsackie virus B in 40 non-diabetic subjects was observed compared with diabetic patients, with no significant difference in levels of antibodies directed toward enterovirus and other viruses (mumps and rubella). Thus, early exposure to these viruses may not be an essential factor in T1DM etiology (Tuveno *et al.*, 2014).

MATERIALS AND METHODS

Study design, area, and population

The study employed a cross-sectional study design, and the study area included General Hospitals in Jigawa State, selecting a representative Hospital from each of the three Senatorial Districts. The Hospitals include: Dutse General Hospital representing Jigawa Central, Hadejia General Hospital representing Jigawa East zone, and Ringim General Hospital representing Jigawa West zone. This research focused on type 1 diabetes patients of all sexes and age groups; therefore, demographic, social, and other data were collected using a structured questionnaire.

Inclusion and Exclusion criteria

All type-1 Diabetes mellitus patients who gave informed consent were included in this study. This study excluded all types of patients except Diabetes mellitus type-1 patients who did not give their consent or those under intensive care.

Sample Size Determination

The sample size has been determined by a Fisher statistical formula for estimating the desired sample size $n=Z^2(p)(q)/[\ d^2\ ]$(Fisher, 1998). Where n=desired sample size, P=prevalence of Enterovirus B among patients with enterovirus infection in Kaduna State, Nigeria (Egboche *et al.*, 2019), q=(1-p), Z=z-factor =1.96, D=allowable error=(0.05). Thus,

$n=(1.96)^2X\ 0.05X\ (1-0.05)/[\ (0.05)^2]$ = 73

n=73 blood samples, and it was increased to 90 in order to have an accurate result.

Screening Type-1 Diabetes using Fasting blood sugar or random blood Sugar test

The screening for TID was carried out using the procedure described by Ahmed *et al.* (2020). Briefly, a blood sample was taken from a patient who had not eaten for at least 8 hours or overnight.

A fasting blood sugar level of 126 mg/dl (7.0mmol/L) or higher suggests type 1 diabetes. Likewise, Random blood sugar was used as a primary screening test for type 1 diabetes. A blood sample was taken at random. A blood sugar level of 200 milligrams per deciliter (mg/dL), or 11.1 millimoles per liter (mmol/L), or higher, along with symptoms, indicates diabetes.

Blood Collection and Serum Preparation

A total of 5ml whole blood was collected from the patients by licensed medical laboratory scientist working in the selected hospitals and transferred into plain container and allowed to clot, and then were centrifuged at 3500 RPM for 10minutes at room temperature and the serum was extracted and some part of the serum was used for ELISA analysis while some were kept at -20°C for molecular analysis.

Detection of Coxsackie B virus IgM using ELISA

This method was employed as described by Ahmed et al. (2020). This confirmatory test for Type-1 diabetes was carried out using blood tests to detect Glutamic acid decarboxylase65 (GAD65), which served as a biomarker in T1DM patients' serum by using an indirect ELISA kit for the detection of IgM (Elabscience, USA). An indirect ELISA kit from the same source was also used for the detection of antibodies against the virus, as described in the procedure provided by the manufacturer.

In a Micro ELISA strip plate, three wells were used as negative controls, two as positive controls, and one as a blank. 100µl of negative and positive controls were added to the respective control wells. The Sample serum was diluted with the Sample diluents at a ratio of 1:10. (100µl of the diluents was added first and then followed by 10µl of the sample) were mixed by gently shaking.

The plate was sealed and incubated at 37°C for 30 minutes. A washing buffer was prepared by Dilute 20 ml of concentrated buffer with 580 ml of distilled water. After incubation, the plate was washed by aspirating and refilling the wells with wash solution, allowing it to stand for 30-60 seconds, and repeating this process 5 times. Next, 100µl of HRP-Conjugate reagent was added to each well except the blank, and the plate was incubated again at 37°C for 30 minutes. After repeating the wash process, 50µl of reagents A and B were added to each well, gently mixed, and incubated for 15 minutes at 37°C. The reaction was stopped by adding 50µl of stop solution. The absorbance of each well was read at 450 nm using a microtiter plate reader.

Result Determination

Positive control average OD ≥ 1.00**,** Negative control average OD ≤ 0.100, and **CUT OFF** value =negative control average + 0.15 and also If OD < CUT OFF: Negative for CBV-IgM, If OD ≥ CUT OFF: Positive for CBV-IgM

Molecular Detection of coxsackie B virus Using RT-PCR

RNA Extraction (Spin Columns cat/RR-C0115)

RNA was extracted according to (Ahmed *et al.*, 2020) by the manufacturer's guide; 2 µl of proteinase k was transferred into 1.5 mililiter microcentrifuge tube, 200 ul of the treated sample was added, followed by 30ul of nucleic acid precipitation aid and then 500 ul of lysis buffer. The mixture was covered and mixed by vortexing 30 seconds and was incubated for 5minutes at room temperature. Then, the mixture was also transferred into a spin column placed in a 2ml collection tube and centrifuged at 12000rpm for one minute; the flow-through was discarded, and then 500µl of washing buffer 1 was added to the spin column and centrifuged 12000rpm for 1minute. The spin column was transferred back into the collection tube and centrifuged at 12000rpm, both the flow throw and the collection tube was discarded. Additionally, the spin column was transferred into a clean 1.5ml tube, and 50µl of the elution buffer was pipetted and directly applied to the silica-based membrane. The sample was then centrifuged for 1 minute to elute, and the extracted nucleic acid was ready for use.

cDNA Synthesis Using RT Easy™ Mix :1.0-1403

cDNA was synthesized according to (Ahmed *et al.*, 2020) by the manufacturer's guide;

10µl of easy mix was added in 1.5ml test tube and 2µl template RNA (specific primer prepared by Africa Genomics Company / Inqaba Biotec West Africa Ltd with Co. Reg. No: RC1232028 and VAT No: 17949735-0001) was added followed by 8 µl of RNase free water was added to the mixture. The mixture was mixed gently by vortexing and centrifuged in order to collect the liquid wet and then inserted into the reaction chamber. 42°C was used for 20 minutes for reverse transcription, and 85°C was used for 5 minutes for inactivation. The reaction was completed, and the synthesized cDNA was used for amplification.

PCR Mixture Preparation
10µl of premix was added to 1.5ml test tube, plus 2µl of template RNA (specific primer prepared by Inqaba Biotec West Africa Ltd with Co. Reg. No: RC1232028 and VAT No: 17949735-0001), and 3µl of cDNA was added, followed by 5µl of distilled water, which gave a 20µl mixture was obtained and was used for PCR

RT-PCR Procedure
Total viral RNA was extracted from serum samples by using RNA extraction kit (spin column Africa Genomics Company / Inqaba Biotec West Africa Ltd with Co. No: cat/RR-C0115). The extracted RNA from serum samples was transferred into 0.2ml tubes. The primers used was designed for conserved region of viral protein 1 (VP1) and was used for direct detection of Human Coxsackievirus B virus at amplicon 471 bp depended on NCBI Genbank and Primer 3plus design, the forward primer was (5' GCAGACCAGACACGTGAAGA-3') and reverse primer was (5'- CATCATAGTCATCAACTTCT-3'), the primers were designed by (African Genomic company). After that, the PCR tubes that contain the entire PCR component were place in a standard PCR Thermocycler (BioRsad/ USA). RT-PCR thermo cycler conditions according to primer annealing temperature and RT-PCR PreMix kit instructions are to be completed in 35cycle and the steps are as follows; Initial Denaturation occurred at a temperature of 95˚C for 5min 1 and final denaturation at a temperature 95˚C for 30sec and then, Annealing occurred at 58˚C for 30sec followed by extension at 72C˚ for 1min and Final extension also occurred at 72C˚ for 5min.Therefore, after PCR amplification, the product was separated on a 1.5% agarose gel through electrophoresis, and the bands were visualized under UV light. An image of the gel was then captured using a Bio-Rad Imaging System.

The agarose gel was made by dissolving 1.5g of agarose powder (1.5%) in 100 mL of 1X TAE buffer, followed by heating and the addition of ethidium bromide®. After solidification, electrophoresis was performed, and the bands were visualized and recorded.

Data Analysis and Result Presentation
Data were analyzed using Statistical Package for the Social Sciences (SPSS) version 20.0 (SPSS Inc, Chicago, IL, USA). The chi-square test was used to examine the association between Coxsackie B virus and Type 1 diabetes. Percentages and tables were used to show the distribution and prevalence of the infection. The level of statistical significance was set as $p< 0.05$

RESULTS

From this finding, out of 90 blood samples tested, a 12.2% seroprevalence of CBV-IgM antibody in Jigawa state was obtained. It was also found that out of 11 CBV-IgM antibody-positive individuals, two were CBV-RNA-positive using RT-PCR, which gives a CBV-RNA prevalence of 2.2%.
Ninety samples were screened in which thirty 30 samples were collected from each Hospital visited as it indicated in Table 1. Dutse General Hospital, representing the Jigawa Central Senatorial District, has a high prevalence of 5.6%, followed by Hadejia General Hospital, representing the Jigawa East Senatorial District, with 4.4%, and then Ringim General Hospital, representing the Jigawa West Senatorial District, with a lower prevalence of 2.2%. The difference in prevalence may be due to differences in the geographical settings of various zones and other factors, such as poor environmental sanitation and other predisposing factors.

Table 1: Serorevalence of coxsackie B virus based on the selected Hospital in the study area

Name of Hospital	No. Tested (%)	No. Positive (%)	No. Negative (%)	*P*-value
Dutse General Hospital	30	5 (5.6)	25 (27.8)	0.484
Ringim General Hospital	30	2 (2.2)	28 (31.1)	-
Hadejia General Hospital	30	4 (4.4)	26 (28.9)	-

Key: No: Number

Out of thirty-two male samples screened, four were positive with a seroprevalence of 4.4%, while seven samples from females were positive with a seroprevalence of 7.7% (Table 2). On the same table it was also computed that IgM antibodies were detected in five blood sample from age group 0-20 years giving a seroprevalence rate of 5.6%, 1 sample was positive in the age group 21-40 years with a seroprevalence rate of 1.1%, 41-60years with a seroprevalence rate of 3.3% and 61-above years

with a seroprevalence rate of 2.2% as in the Table 2.

Out of sixty-two rural resident samples screened, eight were positive with a seroprevalence of 8.9%, while three samples from urban patients were positive with a seroprevalence of 3.3% as given in Table 2 above.

Table 2: Seroprevalence of Coxsackie B virus based on demographic information of the patients

Variable	No. Tested (%)	No. Positive (%)	No. Negative (%)	*P*-value
Sex				
Male	32 (35.5)	4 (4.4)	28 (31.1)	0.599
Female	58 (64.5)	7 (7.7)	51 (56.7)	
Age group (yrs)				
0–20	25 (27.0)	5 (5.6)	20 (22.2)	0.051
21–40	18 (20.0)	1 (1.1)	17 (18.9)	
41–60	30 (33.3)	3 (3.3)	27 (30.0)	
61 and above	17 (18.8)	2 (2.2)	15 (16.7)	
Residency				
Rural	62 (68.9)	8 (8.9)	54 (60.0)	0.154
Urban	28 (31.1)	3 (3.3)	25 (27.8)	

Keys: No.: Number, Yrs: Years

From Table 3 with which indicates the way of acquiring the disease, 45.5% were inherited with prevalence of 7.8% and 54.5% were acquired via environmental exposure as provided with prevalence of 4.4% and also on the same table those with primary education has high prevalence of the virus with 6.7% followed by those with secondary education with 4.4% and the those with tertiary education have less prevalence with 1.1%. Hence, regarding the parental status of the disease, those who's both of their parents have the disease possess a high prevalence with 8.9% followed by those who's acquired the disease from their father only, with 2.2% and those who acquired it from their mother only have 1.1%.

In Figure 1 below, bp represents base pairs, which range from 100 bp to 1500 bp on the DNA ladder. The positive samples among the 11 tested were lanes 6 and 11, while all others were negative. The -ve represents the negative control, and M represents the DNA ladder.

Table 3: Seroprevalence of coxsackie B virus with regard to educational level, mode of acquiring the Disease, and suspected acquired parents

Variables	No. Screened (%)	No. Positive (%)	No. Negative (%)	P-value
Educational Level				**0.279**
Tertiary	18	1 (1.1)	17 (18.9)	
Secondary	29	4 (4.4)	25 (27.8)	
Primary	43	6 (6.7)	37 (41.1)	
Possible Way of Contact				**0.199**
Inherited	41 (45.5)	7 (7.8)	34 (37.8)	
Not Inherited	49 (54.5)	4 (4.4)	45 (50.0)	
Parental Status of the Disease				**0.059**
Both Father and Mother	36	8 (8.9)	28 (31.1)	
Father Only	23	2 (2.2)	21 (23.3)	
Mother Only	31	1 (1.1)	30 (33.3)	

Keys: No.: Number, %: Percentage and Yrs: Years

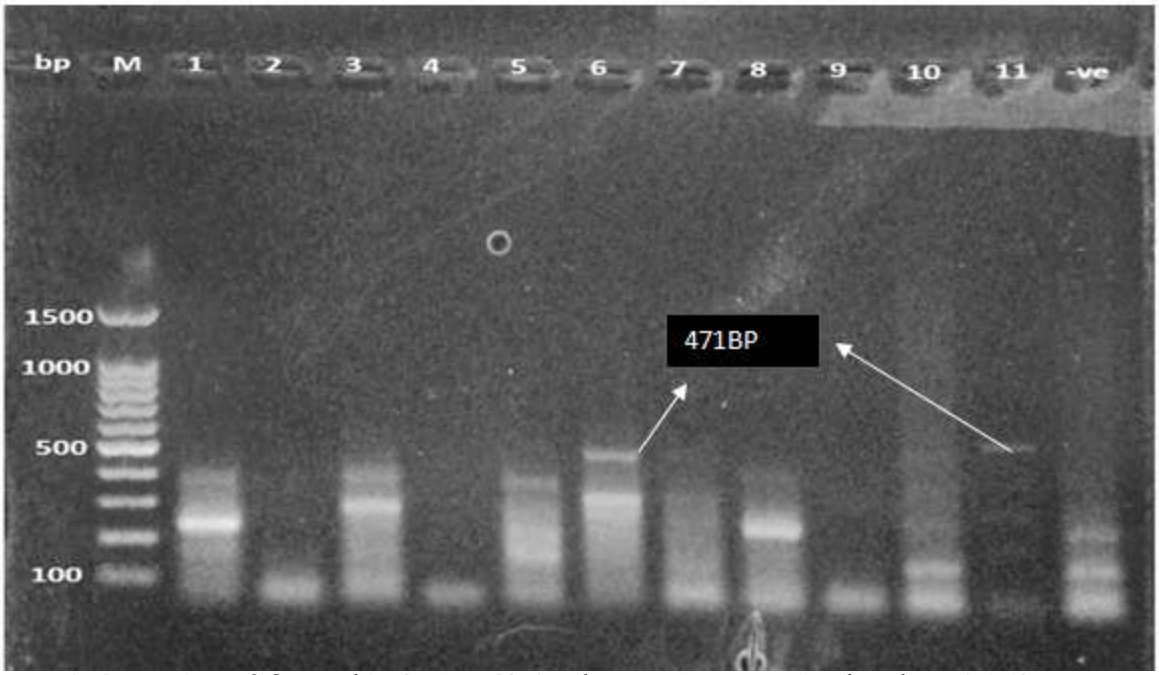

Figure 1: Detection of Coxsackie B virus Vp1 subgenomic gene visualized on 1.0 % agarose gel stained with EtBr and visualized under UV. M is the 100bp DNA ladder, 1-11 stands for the sample number, -ve represents the negative control

DISCUSSION

Using serum samples from 90 diabetic patients that were exposed to both ELISA and molecular identification, this study found that there is a lower prevalence of both CBV-IgM and molecular analysis. A similar incidence was also found in the study conducted by Peter *et al*. (2020) in ten northern states, including Jigawa State is contrary to this finding, a higher prevalence rate of in stool samples screened by RT-PCR for the enterovirus VP1 gene was reported in a research conducted in Ibadan, Nigeria, among children aged 1 to 10 years (Achembach *et al*., 2015). Another study was also conducted in Maiduguri on the prevalence of enterovirus among children aged 0-20 with type 1 diabetes mellitus (T1DM) patients. This data contrasts with the prevalence obtained by Oderinde et al. (2013). The discrepancy may be caused by variations in the research design, the type of sample gathered for analysis during the investigations, the detection procedures, variations in temperature and location, the viral strain, and sanitary practices.

Regarding the global results, a study conducted in Egypt (Bilal *et al*., 2019) on the molecular identification of enterovirus (coxsackie B virus) among T1DM patients also produced similar results. This is because exposure to risk factors have demonstrated a noteworthy correlation with the development of type 1 diabetes during the prenatal and postnatal phases. In a study on non-polio-enterovirus (NPEV) by Camphbel *et al*., (2016), the results from some West African countries were higher than others. For example, the results of The Gambia, Guinea-Bissau, Guinea, Mauritania, Niger, and Senegal are very similar to the study's results in the state of Jigawa, Nigeria, particularly those of The Gambia. When it comes to the prevalence of each group, it shows that the prevalence of male samples is higher than that of female samples.

The results do not align with the findings of a study conducted by Peter *et al*. (2020) in 10 northern states, where the prevalence was high among females. This finding also contrasts with another study in Iraq, which shows that the prevalence of females is higher than that of males. Geographical variation may be the cause of the variation in gender prevalence among the three studies. The highest prevalence was observed in the 0-20 age range, which is inconsistent with the result of Bilal et al. (2019) in Iraq and also differs from the finding of Peter et al. (2020) in ten Northern states, where a high prevalence was observed among the 0-15-year age group.

Regarding residency, it was found that rural areas have a higher prevalence than urban areas. This is because the pathogenesis of Coxsackie B virus, especially among children and adolescents, is more prevalent in rural areas, which can be attributed to poor sanitation and a high level of water contamination.

Enterovirus has frequently existed in irrigation water, ground-water, treated effluents, raw sewage, river water and also in treated waters which resulted in spreading of enterovirus through oral-fecal route and could consider as potential health risk for consumers particularly coxsackies virus which could be transmitted among children with poor hygienic conditions, and also the concentration may found into rural area and that may reflect the high incidence of T1D among these populations Peter *et al.*, (2020). Many studies have revealed that the CVB may represent a more frequently isolated serotype among other enteroviruses in wastewater and contaminated plants. This result is quite different from the finding of Bilal et al. (2019) in Iraq, in which urban areas have a higher prevalence than rural areas.

In this research work, it was observed that a low educational background is directly related to Coxsackie B virus infection, as indicated by the findings. Out of 11 tested samples, six with primary education were found to be positive, with a high prevalence, while only one of those with tertiary education was found to be positive, with a low prevalence. Concerning the parental status of the patient, it was found that patients with a high prevalence of infection in both their father and mother have the infection, whereas patients with a history of infection in their mother or mother only have a lower prevalence. This is due to the fact that the frequency of the disease is higher in close relatives of infected subjects than in the general (non-infected) population. The risk of a member of the general population developing T1D is often quoted as 0.4%. This increases to >1% if the mother has diabetes and intriguingly to >3% if the father has T1D (Wild *et al.*, 2014).

Therefore, as it was revealed samples collected from Dutse General Hospital has high number of positive (five samples) with high prevalence followed by Hadejia General Hospital and then Ringin General Hospital respectively of the coxsackie B virus, the difference may due to the facts that Dutse senatorial zone is the largest senatorial Districts with many villages and the inhabitants of zone are predominantly farmers and exposure to such activities may leads to the easier way to contact the virus and Also by nature of the rural environment little or no attention is given to environmental Sanitation, other occupations typical to rural area are also lead to contacts the virus..

This result shows that the CBV virus's RNA genome is present in the total RNA that was recovered from the plasma of T1DM patients. The prevalence of CBV-RNA in seropositive CVB-T1D patients was very low, according to the results of RT-PCR amplification. In a prior study conducted in Ibadan, Nigeria, among children aged 1–10 years, a higher prevalence was observed for the enterovirus VP1 gene in stool samples screened by RT-PCR (Stanway *et al.*, 2015) is inconsistent with these findings, and the discrepancy between the two results could be the result of different sample types and molecular detection methods.

CONCLUSION

Conclusively, our findings indicate that sociodemographic factors, such as age, educational attainment, parental status, and place of residence, have a substantial correlation with Type 1 diabetes and are responsible for its development, in addition to the Coxsackie B virus. However, Jigawa State generally has a low prevalence (2.2%) of the Coxsackie B virus, as detected by IgM using ELISA in confirmed T1DM patients, suggesting that other factors are responsible for the causation of T1DM.

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