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RESEARCH ARTICLE

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Diversity of enteroviruses in cerebrospinal fluid specimens collected from hospitalised patients in the private and public sector in South Africa

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Abstract

Enteroviruses cause a wide range of neurological illnesses such as encephalitis, meningitis, and acute flaccid paralysis. Two types of enteroviruses, echovirus E4 and E9, have recently been detected in South Africa and are known to be associated with meningitis and encephalitis. The objective of this study was to characterize enterovirus strains detected in cerebrospinal fluid specimens of hospitalized patients in the private and public sector to identify genotypes associated with meningitis and encephalitis. From January 2019 to June 2021 enterovirus positive nucleic acid samples were obtained from a private ($n = 116$) and a public sector ($n = 101$) laboratory. These enteroviruses were typed using a nested set of primers targeting the VP1 region of the enterovirus genome, followed by Sanger sequencing and BLASTn analysis. Forty-two percent (91/217) of the strains could be genotyped. *Enterovirus B* species was the major species detected in 95% (86/91) of the specimens, followed by species *C* in 3% (3/91) and species *A* in 2% (2/91) of the specimens. Echovirus E4 and E9 were the two major types identified in this study and were detected in 70% (64/91) and in 10% (9/91) of specimens, respectively. Echovirus E11 has previously been identified in sewage samples from South Africa, but this study is the first to report Echovirus E11 in cerebrospinal fluid specimens from South African patients. The genotypes identified during this study are known to be associated with encephalitis and meningitis. The predominant detection of echovirus E4 followed by E9 corresponds with other studies conducted in South Africa.

KEYWORDS

cerebrospinal fluid, echovirus, enterovirus, meningitis, South Africa

Institution where work was performed: University of Pretoria.

1 | INTRODUCTION

Enteroviruses are positive sense single-stranded non-enveloped RNA viruses and belong to the genus *Enterovirus*, within the family of *Picornaviridae*.[1] There are 15 species within the enterovirus genus, including, coxsackieviruses, polioviruses, echoviruses, numbered enteroviruses and rhinoviruses.[2] Human *Enterovirus* and *Rhinovirus* species are comprised of *Enterovirus A* to *Enterovirus D* species along with *Rhinovirus A* to *Rhinovirus C* species.[3] Enteroviruses are transmitted via the faecal-oral route or respiratory aerosols, followed by viral replication within the epithelial cells covering the gastrointestinal or respiratory tracts.[4,5] Enteroviruses cause a wide range of neurological illnesses such as encephalitis, meningitis and acute flaccid paralysis.[6] In addition to the neurological complications, enteroviruses can also be associated with myocarditis, herpangina, hand-foot-and-mouth disease, type-1 diabetes mellitus and sepsis-like illnesses.[7–10] Viral aetiologies of encephalitis are diverse and may include enteroviruses, Japanese encephalitis virus and herpes simplex virus.[11,12] Viral meningitis is associated with enteroviruses and herpes simplex virus in addition to West Nile virus, varicella-zoster virus, mumps virus and cytomegalovirus.[11,13,14]

Although a few studies have investigated the types of enteroviruses circulating in South Africa, typing of enteroviruses is not done routinely by diagnostic laboratories.[15–18] In addition, three of the four previous studies were conducted in the Western or Eastern Cape provinces. There is therefore limited epidemiological data on the circulating enteroviruses in South Africa. The objective of this study was to characterize enterovirus strains detected in cerebrospinal fluid specimens of hospitalized patients in the private and public sector to identify genotypes associated with meningitis and encephalitis, using Sanger sequencing and BLASTn analysis.

2 | METHODS

2.1 | Study population

This study was a retrospective and prospective study to determine the prevalence and types of *Enterovirus* species causing encephalitis and meningitis in hospitalized South African patients. From January 2019 to June 2021 a total of 217 enterovirus positive nucleic acid samples were received from diagnostic laboratories in the public (National Health Laboratory Service—Tshwane Academic Division Virology, NHLS-TAD) and private sector (AMPATH laboratory). The NHLS-TAD laboratory mainly receives specimens from hospitals in Tshwane, Gauteng and AMPATH receives specimens from across South Africa.

These nucleic acid samples tested positive for enterovirus by qualitative real-time RT-PCR with a Ct value below 40 and were obtained from cerebrospinal fluid specimens. In addition, nucleic acid was obtained from an opportunistic stool specimen collected from a 9-day-old female patient that tested positive for enterovirus at NHLS-TAD.

2.2 | Viral RNA extraction and detection of enteroviruses

The NHLS-TAD laboratory manually extracted the viral RNA using the QIAamp viral RNA mini kit (Qiagen), followed by detection of enteroviruses using an in-house qualitative real-time RT-PCR assay targeting the 5′-untranslated region described by Nijhuis et al.[19] on a LightCycler® 2.0 (Roche). In contrast, AMPATH performed extraction using an automated EMAG® instrument (bioMérieux), followed by detection using the Multiplex FTIyo Viral Meningitis kit (Fast Track Diagnostics), on a LightCycler® 480 (Roche).

2.3 | Preparation of complementary DNA (cDNA)

The extracted nucleic acids (10 µL) were subjected to RNA denaturation at 95°C for 2 minutes (min) with 2 µL of random hexamer primers (600 pmol/µL) (Roche). This was followed by cDNA synthesis using Protoscript II Reverse Transcriptase (New England Biolabs) according to the manufacturer's instructions.

2.4 | Genotyping of enterovirus positive viral RNA targeting the VP1 region

A nested PCR was performed in 50 µL reactions using 1X EmeraldAmp® MAX HS PCR master mix (Takara Bio Inc.) with primer sets and cycling conditions described by Nix et al.[20] The first PCR was performed with primer set 1 (1 µM each of EV 222 & EV 224) and 1 µL cDNA followed by the nested reaction with primer set 2 (1 µM each of AN 88 & AN 89) and 1 µL of the first round reaction. The primer sequences are shown in Table 1. The first round yielded a PCR

TABLE 1 Primer sequences used for the amplification of the VP1 region of enteroviruses.[20]

EV primers	Sequence 5′–3′	Orientation	Round	Location on genome	Fragment size
EV 224	GCIATGYTIGGIACICAYRT	Forward	1st	1977–1996	992 bp
EV 222	CICCIGGIGGIAYRWACAT	Reverse	1st	2969–2951	
AN 89	CCAGCACTGACAGCAGYNGARAYNGG	Forward	2nd	2602–2627	375 bp
AN 88	TACTGGACCACCTGGNGGNAYRWACAT	Reverse	2nd	2977–2951	

Note: I—Inosine, Y—C or T, R—A or G, W—A or T, N—any base.

product length of 992 base pairs (bp) and the nested PCR a product of 375 bp as detected by agarose gel electrophoresis.

2.5 | Sanger sequencing, cloning and colony PCR

The nested AN 88 and AN 89 primers published by Nix et al.[20] were used for Sanger sequencing in conjunction with the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) according to the manufacturer's instructions.[20] The products obtained were analyzed on an ABI3500XL genetic analyzer (Thermo Fisher Scientific) by Inqaba Biotechnical Industries.

Cloning was performed if faint bands were present after agarose gel electrophoresis or if co-infections were identified after Sanger sequencing. Cloning and colony PCR were performed using the Clone Jet PCR cloning kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Transformation was done using 10 beta *E. cloni* competent cells (New England Biolabs). All nested PCR products and colony PCR products were purified with the DNA Clean and Concentrator kit™− 25 (Zymo Research).

2.6 | Phylogenetic analysis

The sequences were analyzed on an online Enterovirus Typing Tool described by Kroneman et al.[21] and BLASTn to assign genotypes and compare the detected sequences to the enterovirus specific sequences in GenBank.[22] Study strain sequences were aligned with reference strains and closely related enterovirus strains using MAFFT version 7. BioEdit was used for manual adjustment of the alignment.[23] Maximum likelihood phylogenetic trees were constructed with the Kimura-2 parameter setting using the MEGA version 11 software, with 1000 bootstrap replicates to illustrate the relationship between enterovirus genotypes.[24]

3 | RESULTS

3.1 | Patient demographics

Sex and age of the patients were the only information received from the respective laboratories for the patients who tested positive for enteroviruses. The patient demographics varied among the public and private sector. The majority (51%) of the specimens received from the public sector were from patients between birth and 5 years of age. This was in contrast to the private sector specimens where the majority (55%) of the patients were between 18 and 59 years of age. The majority of the public sector specimens were obtained from females (49%), whereas the majority of the private sector specimens were received from males (55%). No data in connection with sex or age were obtained for 19% of the patients in the public sector and 15% of patients in the private sector, as shown in Table S1.

3.2 | Impact of coronavirus disease-19 (COVID-19) pandemic on study sample collection

A total of 217 enterovirus positive RNA extracts (101 public sector; 116 private sector) were included in this study. Nucleic acid samples were only received from AMPATH Laboratories for 3 months (February, March, and April) in 2019. Most of the nucleic acid samples obtained from the NHLS-TAD were from 2019 (January to November) with only a few from 2020 (February, March, and September) (Figure 1). No specimens were received from the public or private sector during the first 6 months of 2021. During the hard lockdown (March 26, 2020 to April 31, 2020, alert level 5) schools and non-essential businesses were closed in South Africa. This was followed by level 4 (May 1, 2020 to May 31, 2020), where schools and non-essential businesses were still not operational. Schools re-opened on

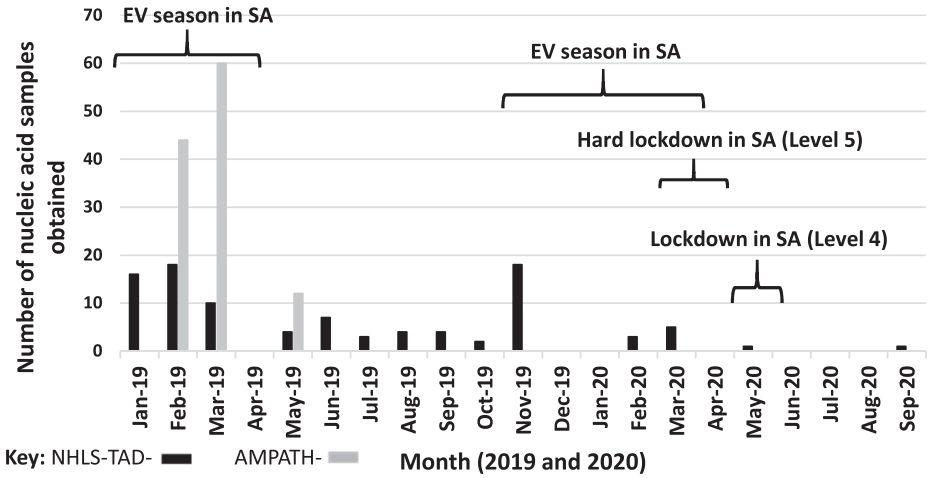

FIGURE 1 Number of nucleic acid samples received from the NHLS-TAD and AMPATH during 2019 and 2020. South African enterovirus seasons and COVID-19 pandemic lockdown periods are indicated with brackets. EV, enterovirus; SA, South Africa.

the 1st of June 2020. Moreover, the wearing of masks was compulsory from the 1st of May 2020 when the country moved to level 4.[25]

3.3 | Genotyping

From the public sector specimens, 25.7% (26/101) of enterovirus strains could be amplified using nested RT-PCR. Of these 89% (23/26) were confirmed as enterovirus after Sanger sequencing and BLASTn analysis. *Enterovirus* species *B* was detected in 78.3% (18/23) of specimens followed by species *A* and *C*, which were detected in 8.3% (2/23) of specimens. Enterovirus C99 was identified in one CSF specimen, while human poliovirus 1 and 3 vaccine strains were detected in a stool specimen of a 9-day-old vaccinated baby. Twelve genotypes detected among the public sector were echovirus E3, E4, E9, coxsackievirus A4, A10, B1, B3, B5, poliovirus 1, 3, and a numbered enterovirus such as enterovirus C99 as shown in Figure 2A. Sixty-two percent (72/116) of enterovirus strains obtained from AMPATH Laboratories yielded the correct product size after nested PCR. Of these, 94% (68/72) of strains were confirmed to be enteroviruses after Sanger sequencing and BLASTn analysis. Eight different genotypes were identified among the 68 confirmed enteroviruses. All of the genotypes detected from the private sector belonged to *Enterovirus* species *B*. These genotypes were echovirus E4, E5, E9, E11, E14, E30 along with a single coxsackievirus B5 (Figure 2A). A larger variety of enterovirus genotypes were identified

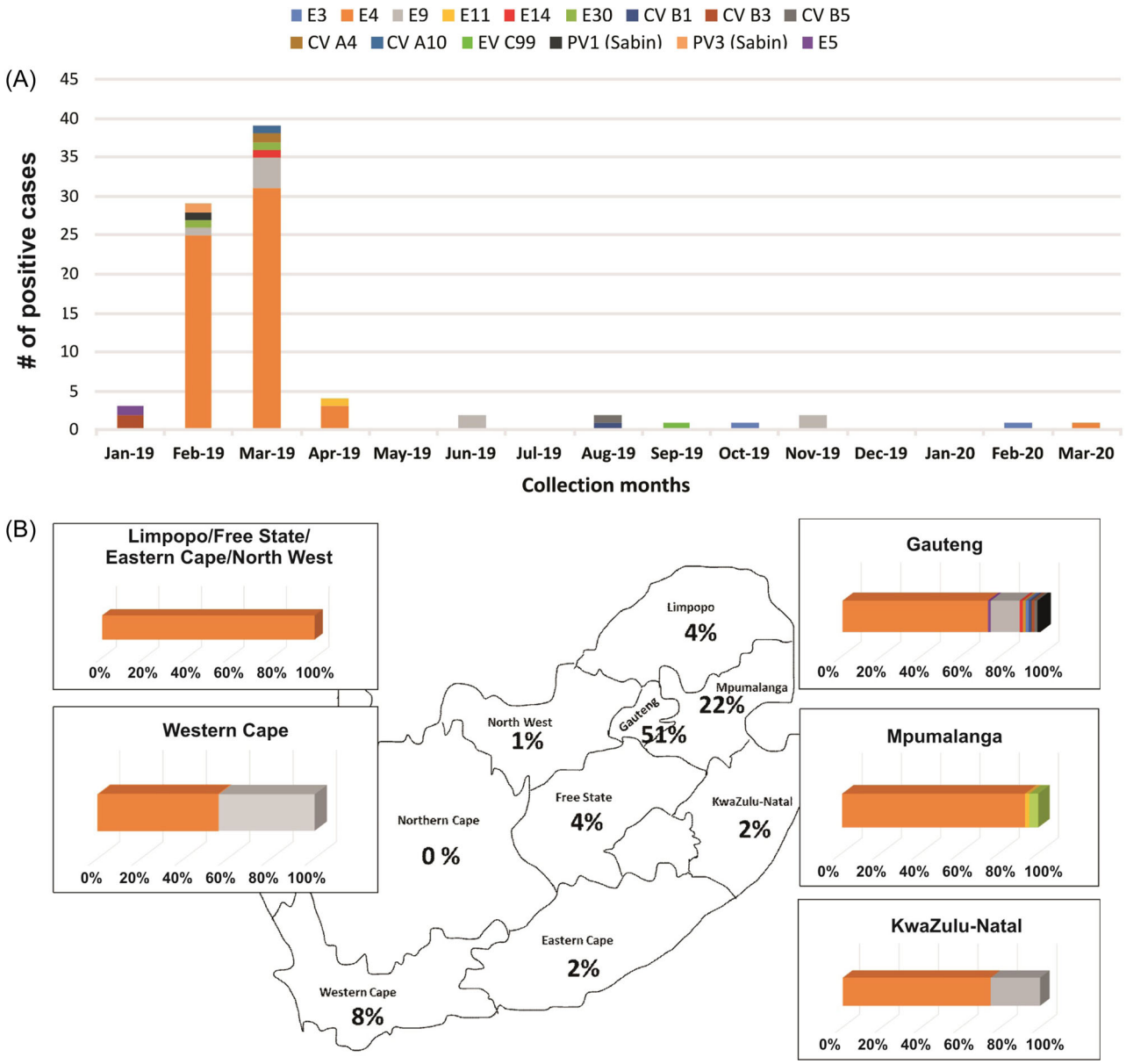

FIGURE 2 (A) Genotypes detected from private and public sector enterovirus positive specimens between 2019 and 2020. (B) Percentage of genotyped strains from each province in South Africa. Image created by author in Microsoft PowerPoint® and Excel.

in the public sector, compared to the private sector. Overall, *Enterovirus B* species was the major species detected in 89% (81/91) (68 from private and 23 from public) specimens, followed by species *C* in 3% (3/91) of specimens and species *A* in 2% (2 of the 91) of specimens. The predominant genotypes observed within the public and private sector were echovirus E4, detected in 70% (64/91), followed by echovirus E9, detected in 10% (9/91) strains. Figure 2B illustrates the origin of the genotyped enterovirus strains from the private and public sector, with the majority (51%) of the genotyped strains identified in the Gauteng province.

3.4 | Phylogenetic analysis

Figure 3 shows the phylogenetic relationship between BLASTn-confirmed enterovirus sequences from the public sector. The

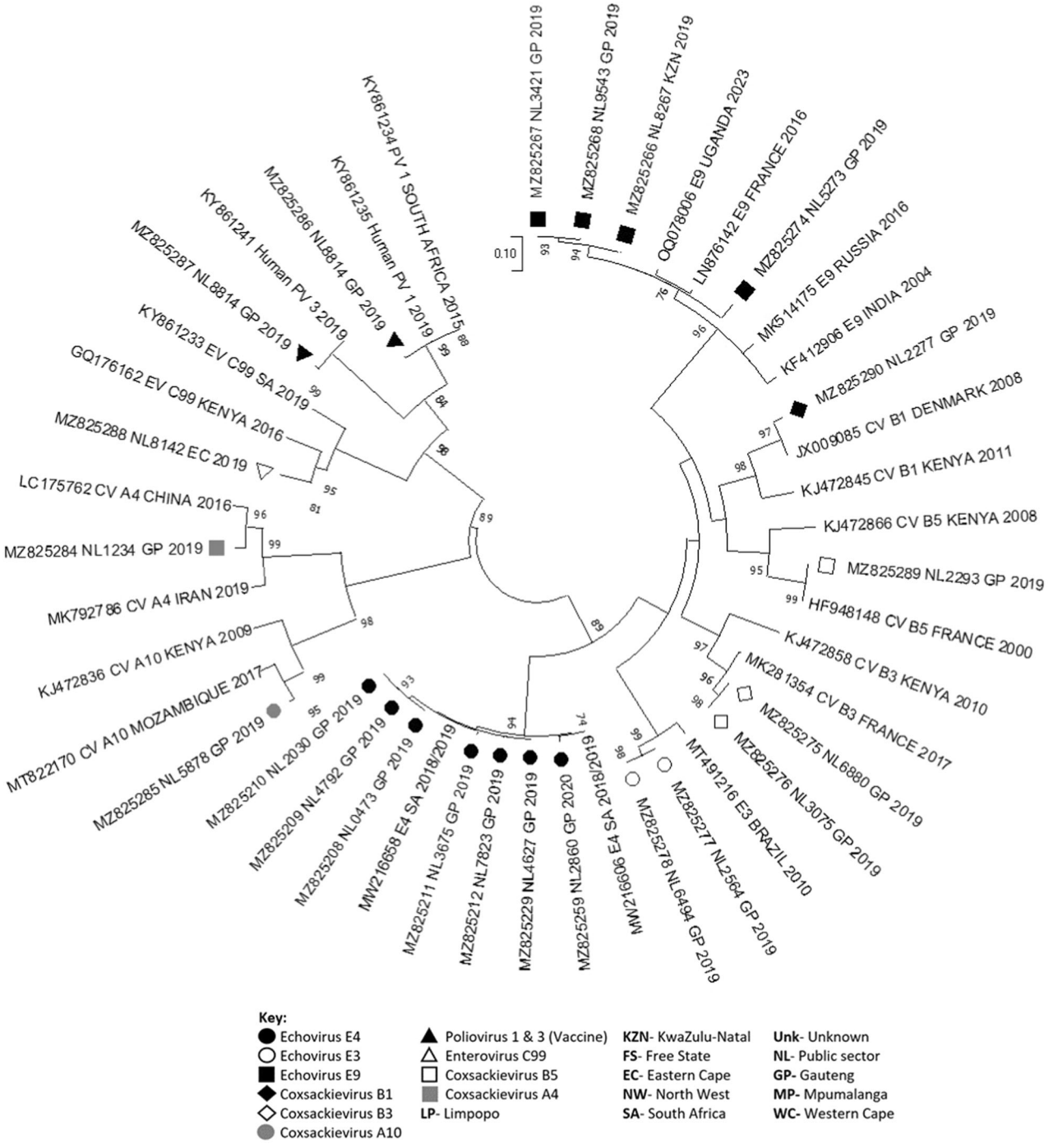

FIGURE 3 The maximum-likelihood phylogenetic tree of enterovirus nucleotide sequences from cerebrospinal fluid specimens from the public sector obtained using Sanger sequencing. The sequences of closely related strains from GenBank were included and are indicated with accession numbers. The length of the sequences used was 266 bp.

coxsackievirus B5 strain was 100% identical to the top hit (HF948148), CVB1, B3, B5 along with E3, E4, and E9 all had more than 95% nucleotide identity over the 375 bp region. Seventy-eight percent of enterovirus positive clinical specimens (public sector) were obtained from academic hospitals in the Tshwane district. Six of the echovirus E4 strains, clustering in the phylogenetic tree, were obtained from the aforementioned hospitals during February 2019 and March 2019 (MZ825208-MZ825212, MZ825229). Although the seventh echovirus E4 strain was detected in March of 2020 (MZ825259), it was closely related to the 2019 strains.

Illustrated in Figure 4 is the phylogenetic relationship between enterovirus nucleotide sequences from the private sector. The

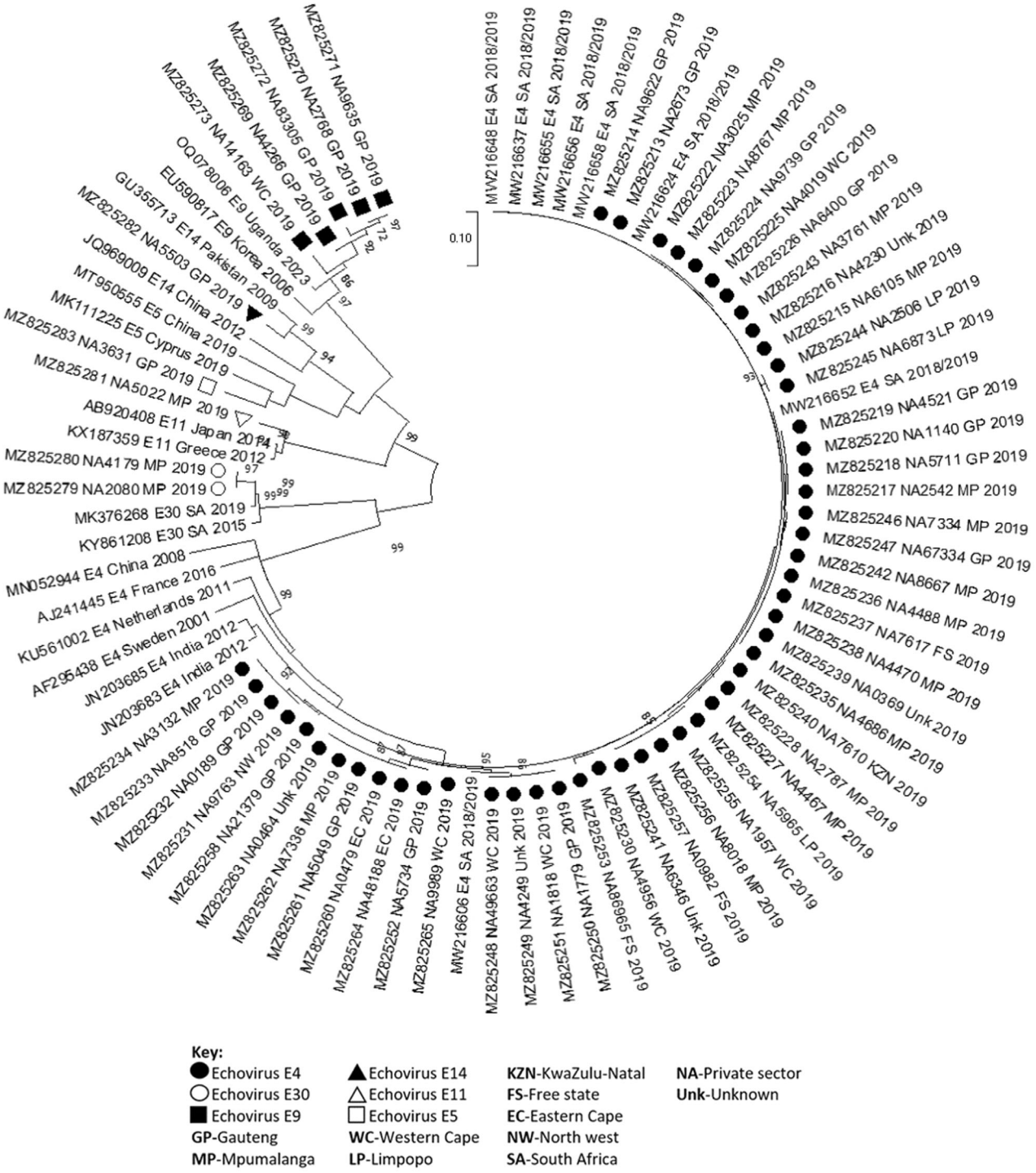

FIGURE 4 The maximum-likelihood phylogenetic tree of enterovirus nucleotide sequences from cerebrospinal fluid specimens from the private sector using Sanger sequencing. Sequences from closely related strains were included from GenBank and are indicated with accession numbers. The length of the sequences used was 266 bp.

majority of enterovirus strains clustered with echovirus E4 reference strains, were highly related to each other (98%–100% nucleotide identity) and to previously identified echovirus E4 strains from South Africa. In contrast, the echovirus E9 strains were most closely related to a strain detected in Uganda in 2012.

Seventeen enterovirus strains from the private sector were received from Mbombela, Mpumalanga Province and were identified as echovirus E4 ($n = 14$), echovirus E11 ($n = 1$) and echovirus E30 ($n = 2$). Table 2 shows the collection date and patient demographic data for the strains detected in patients from Mbombela.

In Figure 5 a maximum-likelihood phylogenetic tree was constructed to evaluate the relationship between the E4 strains obtained during this study and other local and international E4 strains.

4 | DISCUSSION

Overall, 42% (91/217) of enterovirus strains from positive clinical specimens were typed during the course of this study using a set of published primers.[20] *Enterovirus* species *B* was the predominant species detected in 89% (81/91) strains among the private and public sector, with echovirus E4 (70%) and E9 (10%) being the predominant genotypes detected from both sectors. The strains detected during this study can be attributed to sporadic cases and some potential outbreak cases. These findings are consistent with the following studies previously conducted in South Africa. As early as 1981 to 1989, echovirus E4 and E9 were detected by viral cell culture in the Western Cape Province during an outbreak of aseptic meningitis.[15] Furthermore, outbreaks of E4 and E9 occurred in the Tshwane metropolitan area, Gauteng Province, in 2010 and 2011.[16] From December 2015 to January 2016 multiple genotypes such as E5, E9, and E30 were detected in Mossel Bay, Western Cape Province, which coincide with the genotypes detected in this study.[17] More recently, a study conducted in the Eastern and Western Cape Provinces during 2018 and 2019 identified outbreaks of E4 and E9 genotypes.[18] The E4 strains detected during this current study are closely related to E4 strains detected from the cerebrospinal fluid specimens in the study conducted in the Eastern and Western Cape Provinces described by Nkosi et al.[18] (Figure 5). In the current study, the majority of the genotypes identified from the private sector were E4 strains (82.4%) (56/68). From February to April 2019, 17 enterovirus strains originated from Mbombela, Mpumalanga. Interestingly, 14 of these 17 enterovirus strains were found to be E4, which could be due to a possible outbreak (Figure 4) and the remaining three were E30 ($n = 2$) and E11 ($n = 1$) strains. Moreover, another possible outbreak of E4 strains was observed during this study, specifically regarding the public sector specimens (Figure 3). These enterovirus strains are all related to E4 and were all acquired from the Tshwane district during February and March of 2019 (Figure 3). This study reported the first E11 virus from cerebrospinal fluid in South Africa. Previous unpublished studies identified E11

TABLE 2 Accession number, collection date and patient demographic data for the echovirus genotypes from Mbombela, Mpumalanga.

Sample ID	Accession nr.	Genotype	Collection date 2019	Age (years)	Sex
NA 7336	MZ825262	Echovirus E4	12 February	29	F
NA 7334	MZ825246	Echovirus E4	12 February	24	F
NA 2080	MZ825279	Echovirus E30	22 February	15	M
NA 4470	MZ825238	Echovirus E4	24 February	37	F
NA 4467	MZ825227	Echovirus E4	24 February	36	F
NA 8667	MZ825242	Echovirus E4	25 February	30	F
NA 8767	MZ825223	Echovirus E4	27 February	34	F
NA 2542	MZ825217	Echovirus E4	2 March	10	M
NA 8018	MZ825256	Echovirus E4	7 March	11	M
NA 4488	MZ825236	Echovirus E4	8 March	35	M
NA 3025	MZ825222	Echovirus E4	8 March	38	F
NA 4686	MZ825235	Echovirus E4	10 March	31	F
NA 2787	MZ825228	Echovirus E4	14 March	15	F
NA 4179	MZ825280	Echovirus E30	15 March	13	F
NA 3132	MZ825234	Echovirus E4	16 March	19	F
NA 3761	MZ825243	Echovirus E4	17 March	16	M
NA 5022	MZ825281	Echovirus E11	14 April	43	M

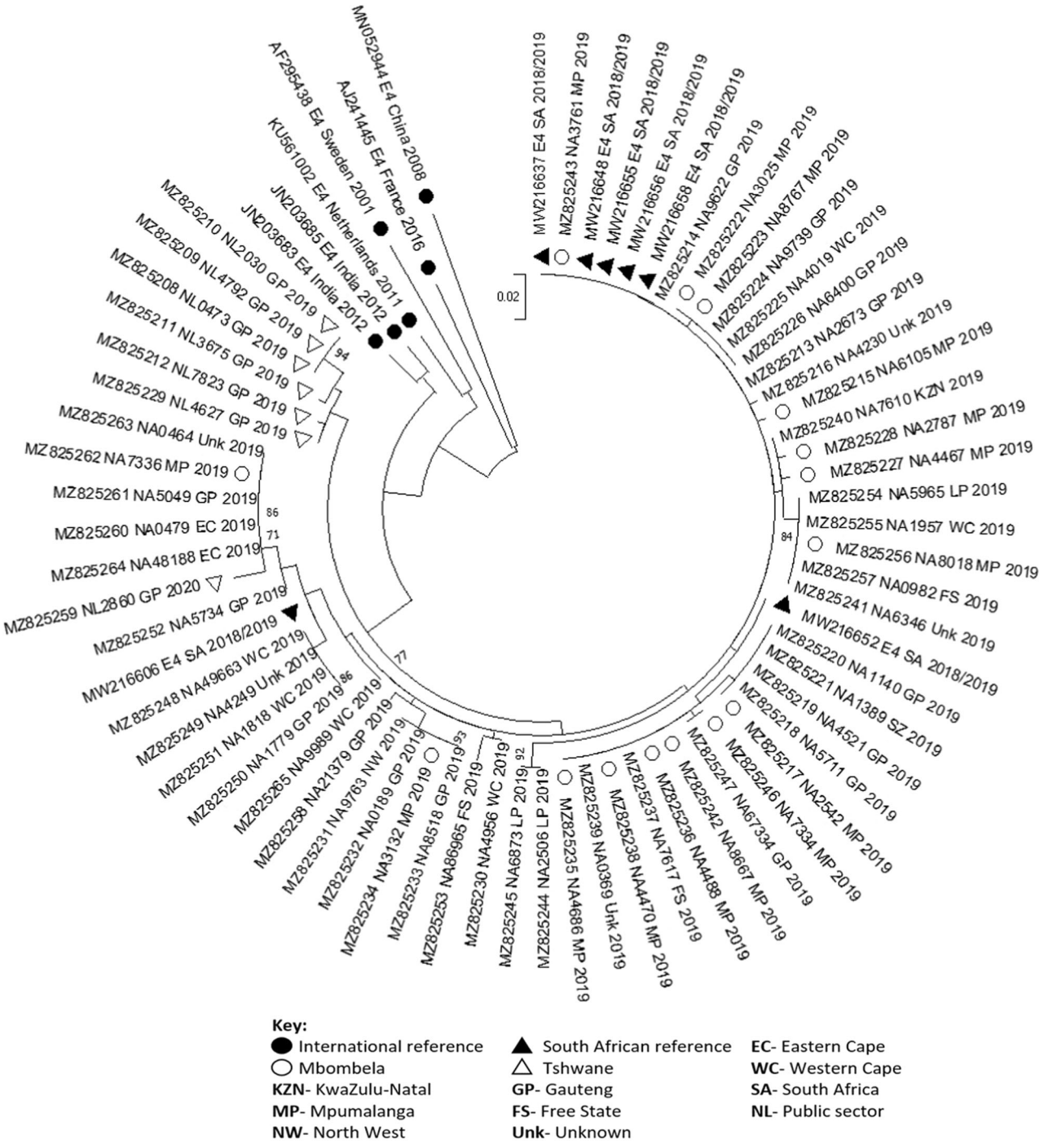

FIGURE 5 Maximum-likelihood phylogenetic tree of Echovirus E4 strains obtained from this study (from each province) compared to Echovirus E4 strains detected nationally and internationally. Strains identified in the Tshwane region of Gauteng (open circle) and Mbombela region of Mpumalanga (open triangle) are specifically indicated. The length of the sequences used was 266 bp.

from sewage and stool specimens in South Africa and submitted sequences to GenBank [AJ871587, KY861194, KY861196]. The E11 isolated from stool/sewage shows no significant similarity to the E11 strain isolated from the CSF. Echovirus E30 is an emerging genotype in European countries (Denmark, Germany, Netherlands, Norway and Sweden) and is widely circulating.[26] In addition to meningitis, E30 often causes mild respiratory disease and non-specific fever, therefore it is not routinely screened for.[27] The E30 strain identified during this study clusters with South African strains (>95% identity) [MK376268; KY861208] and shows less similarity to international strains (<80%). Other genotypes such as coxsackievirus B3 and B5, both clustered with strains detected in Kenya in 2011 (Figure 3).[28] However, Figure 3 shows that the coxsackievirus B3 strain also clustered closely with a strain detected in France in 2017 (GenBank

accession number MK281354).[29] Coxsackievirus B3 has a world-wide geographic distribution.[30] Coxsackievirus B3 is associated with myocarditis in addition to meningitis and most commonly infects infants, which coincides with the age group represented in this study (Median age of 3.5 months; supplementary table).[31]

Three genotypes were detected from *Enterovirus C* species during this study. Two poliovirus vaccine strains were identified from an opportunistic stool specimen received during this study. The third species *C* genotype was enterovirus C99, which was identified from the public sector clinical specimen obtained from the Eastern Cape Province. This enterovirus C99 strain as well as the poliovirus vaccine strains are closely related to an enterovirus C99 and poliovirus vaccine strains identified from a study conducted on wastewater in 2015 in the Tshwane area (unpublished—KY861233) (Figure 3).

A study conducted on the world-wide prevalence and distribution of enteroviruses concluded that the majority of *Enterovirus B* species were detected from cerebrospinal fluid specimens, whereas the majority of *Enterovirus C* species were detected from stool specimens.[32] This information correlates with the findings from this study as the majority of the genotypes obtained from cerebrospinal fluid specimens was from *Enterovirus B* species. Additionally, two out of three *Enterovirus C* species detected during this study was from an opportunistic stool specimen and not cerebrospinal fluid. A study performed to identify the prevalence of enteroviruses in sub-Saharan Africa found that *Enterovirus C* species were not as prevalent as *Enterovirus B* species, but enterovirus C99 was the second predominant genotype detected from faecal specimens from Malawian children in terms of the *Enterovirus C* species.[33] According to Majumdar et al.,[34] *Enterovirus B* species were detected in 84.6% of cerebrospinal fluid specimens compared to the detection of species *B* in blood (83.3%), stool (43.3%), respiratory specimens (10.8%) and skin swabs (no detection). Since *Enterovirus B* species is the major cause of encephalitis and meningitis cases, it is probably more likely to be detected in cerebrospinal fluid. In addition, *Enterovirus B* species is the largest and most prevalent species of the enteroviruses, so the chances of being detected might be higher than with the other *Enterovirus* species.[18,33,35] Last, detection and typing methods could be biased towards *Enterovirus B* species. To eliminate *Enterovirus B* species bias, a cell culture independent detection and typing method was suggested by Nix et al.[20] In the past *Enterovirus B* species bias was noted because viruses from clinical specimens were cell cultured in a Rhabdomyosarcoma cell line, which showed a greater susceptibility to *Enterovirus B* species in comparison to other *Enterovirus* species.[36] However, during the current study no routine cell culturing was performed before the detection and typing of *Enterovirus* species.

The limitations of this study were attributed to multiple factors, some that were out of our control. First, due to COVID-19 pandemic a lower-than-expected number of enterovirus positive clinical specimens were obtained during the study period. This could be explained by a reduction in viral transmission due to the closing of schools, social distancing, increased hand hygiene and wearing of masks. Secondly, this study had a 42% typing success rate attained from extracted nucleic acid samples obtained from cerebrospinal fluid specimens, which might have been due to the long periods of storage of the nucleic acids at −80°C. Another South African study reported a higher typing success rate (59.9%).[17] A previous study reported that typing from faecal specimens had the highest sensitivity compared to cerebrospinal fluid specimens. The same study also indicated that cerebrospinal fluid collected more than 2 days after the onset of clinical symptoms had a lower viral titre compared to cerebrospinal fluid collected in less than 2 days from the onset of symptoms.[37]

Parallel collection and testing of cerebrospinal fluid and stool specimens may improve the genotyping success rate of enteroviruses detected in meningitis cases. Other factors such as transport and sample storage conditions were unknown for the primary cerebrospinal fluid specimens. It would also be beneficial to know during which stage of the disease the specimens were collected. These factors affect the quality of the biological material obtained, which could potentially have an effect on the genotyping efficiency. Another factor that could have had an impact on the genotyping variability would be the time frame between nucleic acid extraction and typing experiments. Prospective studies should conduct sequencing and infer genotypes using whole genome shotgun sequencing to prevent bias.

5 | CONCLUSION

To conclude, most of the genotypes identified during this study are known to be associated with encephalitis and meningitis. Echovirus E4 followed by E9 were the major genotypes detected and this finding corresponds with other studies conducted in South Africa.[15,16,18] Partial amplification and sequencing of the VP1 region using Sanger sequencing is sufficient to type enterovirus genotypes in most cases. However, next generation sequencing will allow for the detection of co-infections of other enterovirus genotypes and other neurotropic viruses.

AUTHOR CONTRIBUTIONS

Megan Janse van Rensburg: Performing laboratory experiments, ethics application, funding application, manuscript preparation. **Janet Mans**: Study conceptualization, ethics application, funding application, technical advice, manuscript review. **Rendani T. Mafuyeka**: Study conceptualization, manuscript review. **Kathy-Anne Strydom**: Specimen collection, manuscript review. **Marcelle Myburgh**: Study conceptualization, specimen collection, manuscript review. **Walda B. van Zyl**: Study conceptualization, ethics application, funding application, specimen collection, technical advice, manuscript review.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The sequencing data that support the findings of this study are openly available in GenBank, reference number MZ2825208 – MZ2825290. No other data are publicly available.

ETHICS STATEMENT

Approval was obtained from the Faculty of Health Sciences Research Ethics Committee of the University of Pretoria under the approval number 265/2020.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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