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Whole Genome Sequence (WGS) of Bordetella pertussis Strains in Amhara Regional State, Ethiopia

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Abstract

Background

Pertussis or whooping cough is an acute respiratory tract disease caused by *Bordetella pertussis*. In 2014, globally, there were an estimated 24.1 million pertussis cases and 160,700 deaths from pertussis in children under five years old. Due to vaccine pressure, there have been genotype changes in the circulating *Bordetella pertussis* strains. This study aimed to determine the genotype distribution of *Bordetella pertussis* strains in Amhara Regional State, Ethiopia.

Methods

A cross-sectional study design was employed on clinically suspected pertussis patients. Sixteen DNA samples positive for IS*481* (Ct ≤ 28 cycles), were sequenced using next-generation Illumina whole genome sequencing. The sequenced data was analyzed using software's (Bowtie2 v2.2.9, Samtools v1.8, Bedtools v2.27.1). Sequenced data was compared with reference strains using Basic Local Alignment Search Tool (BLAST) and phylogenetic tree.

Results

The ptxP1, fim4 and prn1 alleles were found in 11/15 (73%) of sequenced samples. The remaining four samples carried the ptxP3, fim2 and prn2 alleles. None of the samples carrying the ptxP1 allele was found to carry the fim2 or prn2 allele and none of the samples carrying the ptxP3 allele was found to carry the fim4 or prn1 allele.

Conclusions

The ptxP1 allele carrying *Bordetella pertussis* was the commonest strain circulating in Amhara Regional State, Ethiopia. An increased number of ptxP3 strains in Ethiopia indicates the shift of *Bordetella pertussis* from ptxP1 to ptxP3 strains. This ptxP allele shift needs further investigation whether it is associated with the type of vaccine used or not.

Introduction

Bordetella pertussis is a strict human pathogen that causes pertussis (whooping cough), an acute respiratory tract disease particularly in young children [1]. Despite extensive vaccination programs, whooping cough is still endemic in many countries, causing approximately 24.1 million cases and 160,700 deaths in children younger than 5 years in 2014 ([1–3].

Despite high childhood vaccination coverage, nowadays, the numbers of reported pertussis cases is increasing in many developing and developed countries. Several explanations have been suggested for this resurgence, including vaccine type used (including suboptimal vaccines), waning of vaccine-induced immunity, improved laboratory diagnostic tools, increased awareness of the disease and genotype changes in the circulating *Bordetella* species population [4–7].

Pertussis prevalence is increasing because under the pressure of vaccination, altered genotypic strains of *B. pertussis* might have emerged which would be less affected by the immune reaction of vaccinated individuals [8, 9]. For instance, early studies on genotypic strains of *B. pertussis* displayed the same gene variants as the vaccine strains. However, the newer strains are predominantly non-vaccine types. Genetic changes observed in the strains are mostly concentrated in antigenic determinant genes, which encode vaccine components, such as pertussis toxin (PT), filamentous hemagglutinin (FHA), pertactin (PRN), and fimbriae (FIM). For example, the circulating strains in America, Asia, Australia and Europe carry a single nucleotide substitution in the pertussis toxin promoter gene 3 (ptxP3) allele that confers a slight increase in pertussis toxin production [8–10].

Globally, 11 ptxP alleles were found in 1,566 strains. Two ptxP alleles were found to predominate worldwide, ptxP1 and ptxP3. The ptxP3 allele was first detected in a strain isolated in the United States in 1984 [11, 12]. *Bordetella pertussis* strains with ptxP1 predominated (88%) from 1935 through 1990 and were found in lower frequencies (47%) from 1991 through 2004. A reverse trend was observed for the ptxP3 strains (3% from 1935 through 1990 and 52% from 1991 through 2004). From 1935 through 1990, ptxP3 strains were only detected in the Netherlands (3%) and the United States (13%). The only region in which ptxP3 strains were not detected was Africa (countries using whole-cell Pertussis (wP) vaccine), where only ptxP1 strains were found [12].

Previously, there was no standardized procedure to genotype/subtype *Bordetella* species. The best method to genotypes/subtypes used so far was pulsed-field gel electrophoresis (PFGE). Therefore, it has been difficult to directly compare PFGE results between laboratories. Thus, globally, the total number of genotypes/subtypes of *Bordetella* cannot be determined easily. Rather, genotype/subtypes for each country/state can be determined. For example in Cincinnati (USA), 30 subtypes by the enzyme XbaI-PFGE, in Alberta (USA), 14 subtypes by XbaI-PFGE and in Massachusetts (USA), 5 subtypes by using XbaI-PFGE and SpeI-PFGE were identified. In Taiwan, 21 subtypes (P1 to P21) were identified by XbaI-PFGE [8, 13,].

More recently, the wide availability and use of whole genome sequencing has enabled us to study the complete genome of *B. pertussis* from clinical specimens. Genome diversity in B. *pertussis* is measured by single-nucleotide polymorphisms and has been described by the allelic profiles of the acellular pertussis (aP) antigen genes ptxA, PRN, FIM2, and FIM3 and the allele of the promoter of the PT locus (primarily either ptxP1 or ptxP3). The whole genome sequence analysis helps to determine any polymorphisms in the *B. pertussis* genome and mutations that could be responsible for its resurgence and outbreaks [12, 14]. Moreover, the complete genome sequence of *B. pertussis* strains is unavailable in Amhara Regional State and in Ethiopia at large. Thus,

the aim of this study was to determine the genotype distribution of *B. pertussis* strains in the Amhara Regional State, Ethiopia, using whole-genome sequencing.

Methods

Study area and setting

The study was conducted in Amhara Regional State, the second most populous region in Ethiopia. Between July 2018 and February 2019, 515 nasopharyngeal swabs were collected from clinically compatible pertussis patients at the University of Gondar Comprehensive Specialized hospital, Masha hospital in Mekdela Woreda (South Wollo Zone), Ebinat hospital (South Gondar Zone), Debark and Janamora hospitals (both in North Gondar Zone).

Nucleic Acid Extraction

Specimens were collected with Dacron swabs and transported in cold container with dry ice to Medical Microbiology laboratory of the University of Gondar Comprehensive Specialized hospital for DNA extraction. DNA was directly extracted from nasopharyngeal swabs using a commercial DNA extraction kit (QIAamp Minikits, Qiagen, Hilden, Germany) according to the manufacturer's instructions. The DNA was eluted into 100µL volume elution buffer and the extracted DNA was stored at -80°C until PCR amplification. A negative control was run every twelfth procedure during DNA extraction. Extracted DNA samples were transported to Pertussis and Diphtheria Laboratory, CDC, Atlanta, GA, U.S.A for PCR and whole genome sequencing.

Multiplex Quantitative RT-PCR Assay Protocol

DNA amplification was performed in a total volume of 25µl in an optical 96-well plate with the AB7500 Applied Biosystems® thermal RT-PCR System (AB7500 Applied Biosystems, Cummings Center, Beverly, MA, USA). As described by Tatti *et al* [15], the CDC assay uses primer and probe sets for 4 targets in 2 separate reactions to identify *B. pertussis, B. holmesii* and *B. parapertussis*: a multiplex reaction of 3 targets (IS*481*, hIS*1001*, pIS*1001*) and a singleplex reaction of 1 target pertussis toxin subunit1 (*ptx*S1). The multiplex amplification mixture contained 4µl of extracted DNA plus a 21µl master mix. The multiplex RT-PCR protocol used was as follows: Thermal at 50°C for 2 minutes, 95°C for 10 minutes (denaturation); followed by 45 cycles of 95°C for 15 seconds, 60°C for 1 minute (amplification). The *ptx*S1 assay was the same, except amplification was at 57°C. A positive result was defined as a Ct<40 cycles for IS*481*, hIS*1001*, pIS*1001* and *ptx*S1. A Ct value of >40 cycles or no detection was considered negative. DNA samples positive for *B. pertussis* (Ct≤28 for IS*481*) were selected for whole genome sequencing.

The DNA of known reference strains of *B. pertussis* (A639), *B. parapertussis* (F585) and *B. holmesii* (C690) were used as positive controls. No-template controls were included in each PCR run as a negative control. The human Ribonuclease P gene (*RNaseP*) was also detected in a separate reaction to exclude the presence of PCR inhibitors and to ensure the DNA quality of each specimen [15].

Culture-independent Whole Genome Sequencing (CIWGS)

Whole genome sequencing was performed on culture-independent specimens (DNA samples extracted from nasopharyngeal swabs). Sixteen *B. pertussis* strains (which showed Ct<28 for IS*481* in RT-PCR) from different nasopharyngeal swabs were sequenced using the next-generation Illumina Miseq Sequencer. Genomic DNA Library was prepared by using NEBNext Ultra II DNA library preparation kit. Briefly, DNA samples were fragmented by ultrasonication followed by DNA purification, end repair, adaptor ligation, U excision, PCR enrichment (with barcode), Tapestation and RNA bait enrichment. Finally, Streptavidin beads capture, PCR amplification and Miseq loading were conducted prior to whole genome sequencing. The preparation was sequenced using an Illumina MiSeq reagent kit (632 cycles, paired ends) according to the manufacturer's instructions [16].

Data Analysis

Socio-demographic and clinical characteristics were collected using structured questionnaire. The sequenced data was analyzed using software's (Bowtie2 v2.2.9, Samtools v1.8, Bedtools v2.27.1, Samtools, cutadapt, Skesa v3.13.0, Quast v5.0, skesa.fasta, Snippy v3 (or v4), Raxml v8 and iToL v4). Sequenced data was compared with reference strains using BLAST and phylogenetic tree.

Study Approvals

Ethical approval was obtained from the University of Gondar institutional review board (IRB) (Ref. No. O/V/P/RCS/05/376/2017). Each hospital, laboratory and the out-patient department was communicated through written letter obtained from the University of Gondar IRB. Each study subject was informed about the objectives of the study that would contribute towards the understanding of distribution of *B. pertussis* strains in Amhara Regional State and Ethiopia at large. The study subjects were informed that it is their right not to participate or withdraw from the study at any time during the study. Each study subject was requested to give written informed consent. An assent form by parents or legal guardians was completed for anyone less than 18 years. Data taken from each study subject was numerically coded, and results obtained were used only for study purposes and kept confidential.

Results

Socio-demographic Characteristics of the Study Participants

For this study, 16 nasopharyngeal swabs yielding Ct≤28 cycles for IS*481* from five hospitals in Amhara Regional State were included. 11/16 (68.8%) of the study participants were males. 6/16 (37.5%) of the participants were 6-14 years old. Equal numbers of the study participants were rural and urban residents.

Data on the vaccination status showed that 10/16 (62.5%) of the participants were fully/partially vaccinated against pertussis. Of the ten fully/partially vaccinated, 9/10 (90%) of the study participants received the full dose (3 doses) of DTP vaccine (Table 1).

Genotype Distribution of *B. pertussis* Strains

In this study, 16 DNA samples that were positive for *B. pertussis* using RT-PCR (Ct ≤28 cycles for IS*481*) were selected for CIWGS. Six samples were collected from the University of Gondar Comprehensive Specialized hospital followed by five from Masha hospital and three samples from Debark hospital. One sample was from Janamora and another one sample from Ebinat hospital. The genome sequences of these 16 *B. pertussis* strains were compared with the genome sequences of 49 reference strains of the CDC. However, one sample that has poor CIWGS result was excluded from phylogenetic tree construction [Table 2 and Figure 1].

Samples were examined for the presence of ptxP, fim, and prn alleles. In this study, we found the ptxP1 allele in 11/15 samples (73%), unlike strains carrying ptxP3 allele, which is prevalent in Europe and U.SA. Strains carrying the ptxP1 allele such as the strain Tohama I, express lower levels of ptx than the ptxP3 strains, which have become more prevalent in recent years. Whereas, four samples carried the ptxP3 allele. In addition, 73% of the Ethiopian *B. pertussis* strains carried fim4, and prn1; however, 27% of *B. pertussis* was shown to display fim2 and prn2 alleles. Interestingly, none of the samples carrying ptxP1 allele was found to carry the fim2 or Prn2 allele, and none of the samples carrying the ptxP3 allele was found to carry the fim4 or prn1 allele [Figure 1].

Phylogenetic Tree

For this study, only the nucleotides at 598 positions shared by all 64 genomes (15 study plus 49 reference genomes) 'core sites' were used to construct phylogenetic tree. The 11/15 (73.3%) of the samples displaying ptxP1, fim4, and prn1 alleles (Gp14, Gp83, Me10, Me29, Me52, Me65, Me74, Ja02, De07, De 29 and Eb28) were found clustered together in the phylogenetic tree whereas the remaining four samples displaying ptxP3, fim2 and prn2 alleles (Gp28, Gp31, Gp34 and Gp50) were found far from the eleven samples but clustered together in the tree [Figure 1].

In the phylogenetic tree, most of samples in this study, 11/15 (73%) were closer to the reference strains B228-CP026465 and B226-CP016957 (both strains from U.S.A) whereas the strains displaying ptxP3, fim2 and prn2 were close to reference strains H710-CP011236 and H866-CP011710 (both strains from U.S.A) [Figure 1]. Both distinct strains (ptxP1 and ptxP3) were detected in samples from the University of Gondar Comprehensive Specialized hospital.

Discussion

In Ethiopia, similar to many other countries with routine vaccination programs against pertussis using wP vaccine, the disease is far from eradication, and the number of cases has been increasing. The hypothesis for the rising prevalence is that under vaccine pressure (especially to aP) *B. pertussis* may adapt slowly via the selection of mutant strains [12]. In our study, the ptxP1 allele found in 11/15 (73%) samples and the remaining four samples (27%) carried the ptxP3 allele. In addition, 73% of the samples were fim4, and prn1 allele displaying strains whereas, 27% of *B. pertussis* strains were shown to carry fim2 and prn2 alleles.

Globally, 11 ptxP alleles were found in 1,566 strains. Two ptxP alleles were found to predominate worldwide, ptxP1 and ptxP3. The ptxP3 allele was first detected in a strain isolated in the United States in 1984 [11]. *Bordetella pertussis* strains with ptxP1 predominated (88%) from 1935 through 1990 and were found in lower frequencies (47%) from 1991 through 2004. A reverse trend was observed for the ptxP3 strains (3% from 1935 through 1990 and 52% from 1991 through 2004). From 1935 through 1990, ptxP3 strains were only detected in the Netherlands (3%) and the United States (13%). The only region in which ptxP3 strains were not detected was Africa (From 1935 through 1990), where only ptxP1 strains were found [12].

In this study, seven patients *B. pertussis* carrying ptxP1 allele were fully vaccinated and the remaining four were unvaccinated. Furthermore, two out of the four patients with ptxP3 strains were fully vaccinated and one was unvaccinated, and the remaining one patient was partially vaccinated. The increased number of ptxP3 strains in Ethiopia indicates that the shift from ptxP1 to ptxP3 strains. The differences in ptxP allele frequencies may be due to differences in vaccines, geographic factors, vaccination history, and vaccination coverage [12]. Nevertheless, these data provide strong evidence that, in most parts of the world, ptxP3 strains emerged recently and replaced the resident ptxP1 strains [12, 14].

As hypothesized earlier, under vaccine pressure *B. pertussis* may be changed to vaccine resistant strains by modifying the vaccine targets. In western countries, the typical example is the rising prevalence of the virulent ptxP3 strains with a predicted higher expression of PT and spreads rapidly [12, 14]. The ptxP3 allele confers increased binding of BvgA compared to ptxP1, resulting in increased toxin production. When compared with ptxP1 strains, ptxP3 strains produce 1.62 times more PT [12]. However, in our study, where wP vaccine is solely used, most (73%) of the samples tested were ptxP1 which is expected to produce less amount of PT. The remaining four samples were carried ptxP3 allele and this number is too small to draw a conclusion for selective vaccine pressure resistant strains.

Before two decades, the detection of ptxP3 allele was restricted to developed countries where aP was used however, in developing countries ptxP1 was the main allele reported in which wP vaccine used [11, 12]. Surprisingly, in Ethiopia, where only wP vaccine used, the finding of ptxP3 was a new information. In this study, one out of the four samples carried ptxP3 allele. Thus, this data does not confirm whether the emergence of this strain was due to wP vaccine pressure or not and needs further similar studies. In western countries the existing aP vaccine was becoming less effective due to the development of vaccine resistant strains [12]. However, in Ethiopia, it needs further in-depth analysis if the aP vaccine is going to replace wP in the future in the face of increasing ptxP3 strains. Furthermore, large-scale studies should be conducted to gather information on how this ptxP3 allele carrying strains affect pathogenesis, diagnosis and vaccine effectiveness pertussis.

Pertactin, one of the *Bordetella* adhesion molecules and involved in resistance to neutrophil-induced bacterial clearance, and component of all aP vaccine, has been recently associated with several outbreaks [19, 21]. The increase in the prevalence of prn2 *B. pertussis* strains is associated with the re-emergence. However, the strains found in Ethiopia carried the prn1 allele, similar to the strains in Australia [17] and Japan [18] that carry the prn1 allele. Contrary to our strains carrying prn1 allele, most *B. pertussis* strains from Argentina and France [19, 20], USA [21], the United Kingdom [22], the Netherlands [23], Poland [24], Finland and Sweden [25], and Russia [26] carried the prn2 allele.

Conclusion

This study is the first to reveal the genotype distribution of *B. pertussis* strains in Amhara Regional State, Ethiopia. The ptxP1 allele was found in 11/15 (73%) samples, and the remaining four of the samples (27%) carried the ptxP3 allele. In addition, 73% of the samples were fim4, and prn1 allele displaying strains. Furthermore, 27% of *B. pertussis* strains were shown to display fim2 and prn2 alleles. The increased number of ptxP3 strains in Ethiopia indicates that the shift from ptxP1 to ptxP3 strains. This ptxP allele shift needs further investigation whether it is associated with the type of vaccine used or not.

Abbreviations

Avg
Average
aP
Acellular Pertussis
BLAST
Basic Local Alignment Search Tool
CDC
Centers for Disease Control and Prevention
Cov
Coverage
Ct
Cycle Threshold
CIWGS
Culture-independent Whole Genome Sequencing
De
Debark
Eb
Ebinat
FHA
Filamentous Hemagglutinin
Fim
Fimbriae
Frac
Fraction
Gp
Gondar
hIS
holmesii Insertion Sequence
IRB
Institutional Review Board
IS
Insertion Sequence
Ja
Janamora
Me
Masha
PCR
Polymerase Chain Reaction
RT
real-time
Prn
Pertactin
PFGE
Pulsed-field Gel Electrophoresis
pIS
parapertussis Insertion Sequence

PT
Pertussis Toxin
PtxP
Pertussis Toxin Promoter
PtxS
Pertussis Toxin Subunit
RNaseP
Ribonuclease P Gene
wP
Whole-cell Pertussis

Declarations

Ethics approval and consent to participate

This study was ethically approved by the University of IRB (Ref. No. O/V/P/RCS/05/376/2017). Each hospital, laboratory and the out-patient department was communicated through written letter obtained from the University of Gondar IRB. In accordance with the 1964 declaration of Helsinki, each study subject was informed about the objectives of the study that would contribute towards the understanding of distribution of *B. pertussis* strains in Amhara Regional State and Ethiopia at large. The study subjects were informed that it is their right not to participate or withdraw from the study at any time during the study. Each study subject was requested to give written informed consent. An assent form by parents or legal guardians was completed for anyone less than 18 years. Data taken from each study subject was numerically coded, and results obtained were used only for study purposes and kept confidential.

Consent for publication

Not applicable.

Availability of data and material

The original data for this study is available from the corresponding author.

Competing interests

The authors declare that they have no competing interests.

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Authors' Contributions

ST* conceptualized and designed the study, carried out data collection, PCR assay and CIWGS. ST*, DD, BT, BG and FM analyzed and interpreted the data, drafted the manuscript and critically reviewed the manuscript.

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Tables

Table 1: Socio-demographic characteristics and vaccination status of study participants, Amhara Regional State, Ethiopia, 2019.

Variable	Category	Frequency	Percent
Gender	Male	11	68.8
	Female	5	31.2
Age group	≤1 year	3	18.8
	1-5 years	5	31.2
	6-14 years	6	37.5
	≥14 years	2	12.5
Residence	Rural	8	50.0
	Urban	8	50.0
Educational status of participants	Preschool	8	50.0
	Illiterate	2	12.5
	Grade 1-4	5	31.2
	12+	1	6.3
Vaccination status	Vaccinated (fully/partially)	10	62.5
	Un-vaccinated	6	37.5
Vaccination (Complete/Incomplete)	Complete (3 doses)	9	90.0
	Incomplete (<3 doses)	1	10.0

Table 2: Characteristics of the 16 DNA samples after CIWGS run for the analysis of the genotype of *B. pertussis* strains.

ID No	Reads-Total	Reads-Human	Frac-Human	Reads-B.p	Frac-B.p	Reads-Unmap	Frac-Unmap	Avg-Cov-B.p	Breadth-20x B.p	Breadth-20x-Frac	Breadth-50x B.p	Breadth-50x-Frac	Bread 100x
De07	2918035	53407	1.83	1096399	37.57	1768229	60.59	75.1589	3264222	79.53	1774735	43.24	55015
De07	5710753	20738	0.36	4086005	71.54	1604010	28.08	469.2049	3977005	96.89	3771941	91.9	34342
De29	3917549	9470	0.24	2151409	54.91	1756670	44.84	221.7112	3932402	95.81	3569780	86.97	29145
De30	2553442	5743	0.22	680157	26.63	1867542	73.13	34.557	276510	6.73	179742	4.37	12417
De30	3540523	48017	1.35	783174	22.12	2709332	76.52	75.5659	2421119	58.99	1079579	26.3	37629
Eb28	3343187	28160	0.84	2863187	85.64	451840	13.51	311.4481	4069034	99.14	3953460	96.32	36001
Gp14	2248787	774939	34.46	1120380	49.82	353468	15.71	129.8264	3973174	96.8	3378750	82.32	20328
Gp28	2622980	9696	0.36	1183717	45.12	1429567	54.5	101.0682	3686406	89.81	2706451	65.94	12351
Gp31	2282841	6266	0.27	1073190	47.01	1203385	52.71	62.4681	1591096	38.76	384276	9.36	17653
Gp31	2529498	4736	0.18	1522865	60.2	1001897	39.6	182.8453	3797923	92.53	3241453	78.97	23983
Gp34	2029805	524350	25.83	770810	37.97	734645	36.19	54.5437	1513249	36.87	282139	6.87	15866
Gp34	3878577	223011	5.74	2042456	52.65	1613110	41.59	213.0946	3919453	95.49	3486936	84.95	26714
Gp50	3414670	1197619	35.07	1263506	37	953545	27.92	33.3983	601664	14.65	116126	2.82	51089
Gp50	1795483	41335	2.3	844374	47.02	909774	50.67	69.0523	2890849	70.43	862838	21.02	32924
Gp83	3319609	831378	25.04	2298015	69.22	190216	5.73	250.6253	4016717	97.86	3788892	92.31	33084
Ja02	3069727	275274	8.96	2067978	67.36	726475	23.66	221.0984	4022444	98	3782327	92.15	32231
Me10	3055897	572785	18.74	1684741	55.13	798371	26.12	175.0605	4013833	97.79	3605946	87.85	25463
Me10	1793637	9694	0.54	1176307	65.58	607636	33.87	132.7565	3688389	89.86	3046433	74.22	21284
Me29	2706133	675572	24.96	1859968	68.73	170593	6.3	235.2003	4064961	99.04	3924906	95.63	34448
Me29	2112380	45562	2.15	1863133	88.2	203685	9.64	220.4661	3882983	94.6	3487577	84.97	28468
Me52	6186735	770353	12.45	5272103	85.21	144279	2.33	529.0803	4077575	99.34	4032089	98.24	38696
Me65	3546272	768809	21.67	2697352	76.06	80111	2.25	286.0531	4017532	97.88	3817979	93.02	34080
Me74	2891538	726041	25.1	1941178	67.13	224319	7.75	232.3796	4055672	98.81	3890540	94.79	33830
Me74	4261999	26973	0.63	3575438	83.89	659588	15.47	407.0803	3994962	97.33	3784959	92.22	34214

Frac= Fraction; B. p= *Bordetella pertussis*; Avg=Average; Cov=Coverage

Figures

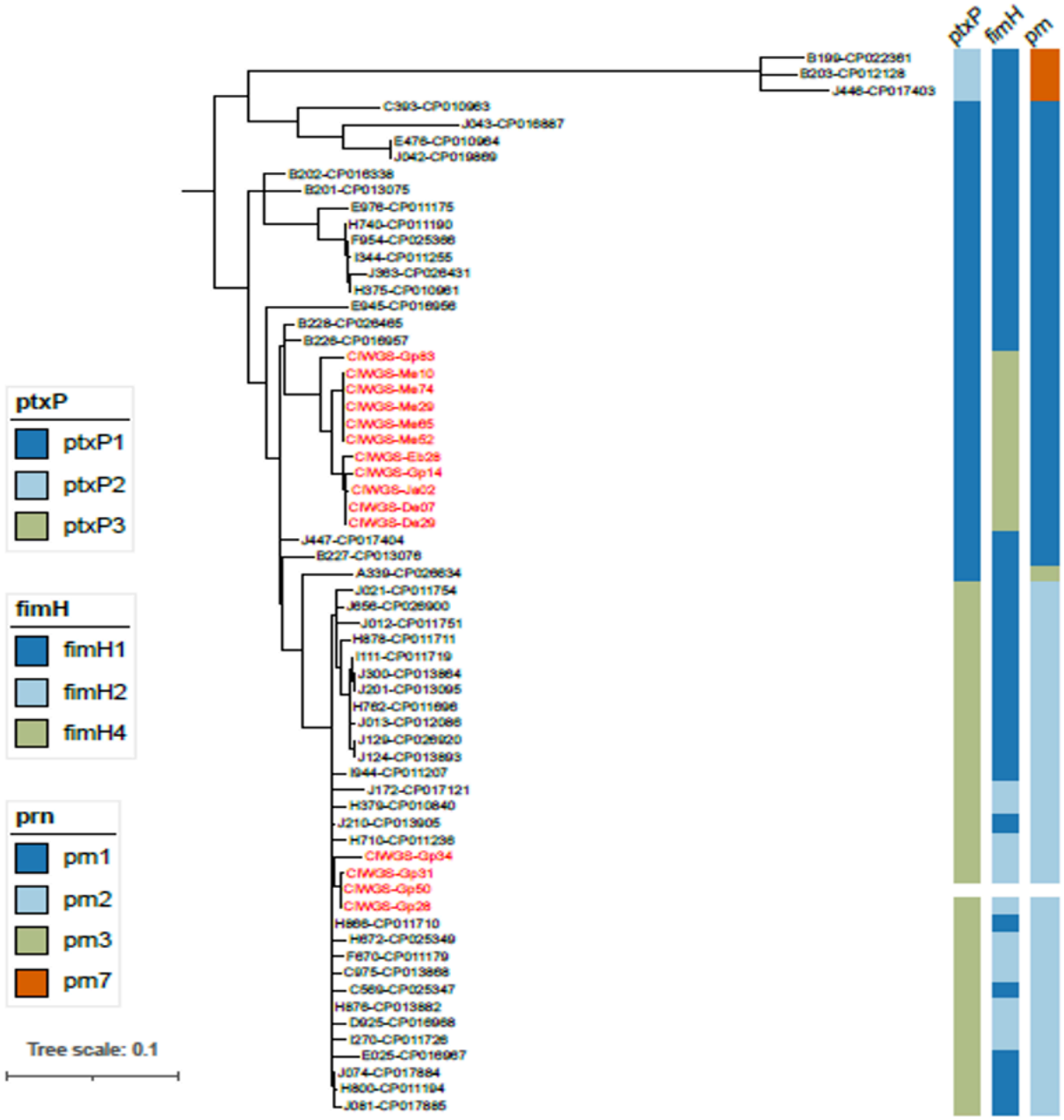


Figure 1

Phylogenetic tree of Ethiopian *B. pertussis* strains in Amhara Regional State, Ethiopia, 2019. ptxP, fimH and prn were shown in light green, deep blue and light blue colors in relation to the reference strains in the phylogenetic tree

ptxP=pertussis toxin promoter; fimH=fimbriae H; prn=pertactin; Eb= Ebinat; De= Debark; Gp= Gondar; Ja= Janamora; Me= Masha