Recent Trends of Antigenic Variation in *Bordetella pertussis* Isolates in Korea

So-Hyun Kim, Jin Lee, Hwa Young Sung, Jae Yon Yu, Seong Han Kim, Mi Sun Park, and Sang-Dun Jung

INTRODUCTION

*Bordetella pertussis* is the pathogenic organism responsible for pertussis. Infants younger than 6 months old are a high-risk group (1). As vaccination can prevent pertussis, the global incidence of reported pertussis cases was dramatically reduced after the introduction of vaccination. However, continuous pertussis outbreaks in countries where vaccination is standard have given rise to major concerns about the control of the disease (2). Various studies suggested that the waning of vaccine immunity (3-5) and the emergence of genotype variants are responsible for the outbreaks (6-8). To improve herd immunity in adolescents and adults, an adult pertussis vaccine was introduced, and vaccination was recommended (9, 10). Genetic changes observed in the strains were mostly concentrated in antigenic determinant genes, which encode vaccine components, such as pertussis toxin, filamentous hemagglutinin, pertactin, and fimbriae (11, 12). More recently, another variant type was also reported (12). This variant was inferred as a more toxic strain because the production of pertussis toxin was increased 2-3 times by a point mutation in the promoter region (13).

In Korea, the number of reported cases of pertussis started to increase from 2009 onward. As shown in Fig. 1, the number of reported pertussis cases was relatively constant during 2000-2008. At this stage, average number of reported pertussis cases was 11.5 cases/yr. However, the reported number of cases rapidly increased to 66 in 2009. At that time, we analyzed the genotype variations to confirm whether there were genotype changes in the strains and we reported the emergence of ST2 strains, which accounted for 58.3% of the strains (14). After the publication of that report, the numbers of pertussis cases increased to 97 cases in 2011 and to 230 cases in 2012. Therefore, we further analyzed genetic variations in 21 strains isolated in 2011-2012 by multilocus sequence typing method. In the present study, we present the results of the comparison of the strains isolated in 2009 with those isolated in 2011-2012.

MATERIALS AND METHODS

Bacterial isolates and culture conditions

Twenty-one *B. pertussis* strains isolated between 2011 and 2012 in Korea were analyzed (Fig. 1). These clinical strains were directly isolated from nasopharyngeal swab or sputum. These specimens were received from hospitals as part of public diagnostic services of pertussis or from affiliated hospitals as part of a pertussis surveillance network. These specimens were directly
inoculated on Reagan Lowe medium (10% horse blood) with cephalixin and incubated in a humid incubator at 37°C for 7 days. After incubation, a suspected colony was confirmed by the slide agglutination test with *Bordetella* antiserum (Difco), and also confirmed by PCR with species-specific primer sets (BP and pTfP) as described in our previous report (14). All the isolated strains were preserved in a deep freezer (-80°C) until analyzed. In addition to these clinical isolates, 3 reference strains (ATCC 10380, ATCC 9797, and Tohama I) were included. The genomic DNA of the isolates was prepared with a commercial kit (GenEx genomic Sx kit, GenaAll Biotechnology, Seoul, Korea) according to the manufacturer’s instruction.

Genotype analysis of target genes
The target genes for MLST analysis were 7 housekeeping genes (adenylate kinase, fumarate hydratase class II, aromatic aminotransferase, isocitrate dehydrogenase, cytosol aminopeptidase, and phosphoglucomutase) and 10 antigenic determinant genes (pertussis toxin subunit 1, pertactin, filamentous hemagglutinin, fimbriae2, fimbriae3, outer-membrane protein q, *Bordetella* antiporter-protein c, adenylate cyclase toxin, virulence-activated gene, and tracheal colonizing factor). The PCR primers and PCR conditions of most of the test genes were those cited in our previous report (14) and in the *Bordetella* multilocus sequence typing web site (http://pubmlst.org/bordetella/). After amplification of the target genes, their nucleotide sequences were confirmed by sequencing.

The genotype determination of the target genes was performed as described in our previous report (14). Briefly, for the housekeeping genes, the sequence information of the tested strains were queried using the *Bordetella* MLST database. For the antigenic determinant genes, the sequences of the tested strains were compared with the DNA sequences of each reported genotype in the NCBI GenBank by the MEGA program (15).

**Multilocus sequence type analysis**
For the sequence types of the housekeeping genes, the genotype profile of each isolate was queried using the *Bordetella* MLST database. For the antigenic determinant genes, we independently assigned sequence types (STs) according to the frequencies of genotype profiles as described in our previous report (14). The relatedness among each ST was analyzed by the START program package (16). The evolutionary relationship and clonal complexes (CCs) among the STs were analyzed and represented as a minimum spanning tree using the PHYLOViZ program (17).

**Analysis of pertussis toxin promoter variations**
To confirm the sequence variations in the pertussis toxin promoter region, a 550 bp target region was amplified using a specific PCR primer set (forward: 5’-AATCGTCCTGCTCAACCGCC-3’, reverse: 5’-GGTATACGGTGGCGGGAGGA-3’) (13). After the PCR, the amplified PCR products were sequenced. The confirmed nucleotide sequences were compared to the reference sequences listed in GenBank (13) using alignment.

**RESULTS**

**Genotype profiles of housekeeping genes**
According to the *Bordetella* MLST database, 43 STs and 4 CCs were observed in the *Bordetella* genus (3 species, *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*) (18). Of these 4 CCs, CC2 belongs to the *B. pertussis* species and is composed of 3 sequence types, ST1, ST2, and ST24. Three similar genotype profiles of 7 housekeeping genes containing these 3 STs (ST1, ST2, and ST24) were observed in this study by querying the *Bordetella* MLST database. Among these STs, the most high frequent was ST2 (Table 1), and it was continuously recorded since 1999. However, frequencies of the other 2 STs (ST1 and ST24) were very low (Table 1) and these 2 STs observed in only old strains. As shown in Fig. 2, ST2 was confirmed as a major ST in the isolates from 2011-2012, with all the tested strains isolated in this period showing a relatively narrow fluctuation range in the difference of annually reported cases. The increasing stage is the period that showed a continuous increase in number of reported cases.
Genotype profiles of antigenic determinant genes

In the analysis of the antigenic determinant genes, 8 STs were confirmed in the Korean isolates (Table 1). For this, the new genotype results confirmed in the isolates of 2011 and 2012 were combined with our previous results (14). The most frequent form was ST1 (57.8%), but this ST was not observed in isolates of 2010. The next frequent type was ST2 (23.5%), which was recognized 2009, was observed until 2012. As shown in Fig. 2, the STs confirmed in the isolates of 2011-2012 were ST2, ST3, and ST8. As reported earlier, ST8 first emerged in this period (14). In addition, the frequency of ST2 increased from 8.6% to 81.0% (P < 0.001). The increase was attributed to an increase in ST2 in the isolates of 2011-2012.

Lineage assignment analysis of antigenic determinant genes

Compared to the results of the analysis of the housekeeping genes, the antigenic determinant gene group showed a more divergent tendency. Only 3 STs were confirmed in the housekeeping gene analysis, whereas 8 STs were confirmed in the analysis of the antigenic determinant genes (Table 1). In addition, although a single CC was confirmed in the antigenic determinant gene analysis, there appeared to be two subgroups, which originated from two central STs. In our previous report (14), we assigned these two subgroups to ACC I and ACC II. As depicted in Fig. 3, all the STs were grouped as a single CC, except ST4 (singleton). However, this single CC was divided into two subgroups, and these two subgroups were linked by two central STs such as ST1 and ST3.

These two subgroups could be characterized by isolation year. As shown in Fig. 4, the strains in the ST1 subgroup (ACC II) were frequently circulated in stable stage (2000-2008, Fig. 1). However, these circulated strains were changed to ST3 subgroup (ACC I) in increasing stage (2009-2012, Fig. 1).

Characteristics of the ST3 subgroup

As shown in Fig. 4, ST3 expanded and ST2 diverged from ST3 in 2009. From 2011, ST2 expanded, and ST8 diverged from ST2. In particular, the frequency of ST2 increased substantially. In this dynamic period of change in the ST of the circulating strains,
reported numbers of pertussis cases also increased considerably (Fig. 1). To shed light on the relationship between the ST2 strain and the increase in pertussis cases, we further characterized the strains isolated in this period about promoter variation. As described, there were reports (12, 13) of more toxic strains showing increased pertussis toxin production due to a mutation in the promoter region (ptxP3 type). As expected, the ptxP3 type was first detected in 2009. The frequency of ptxP3 gradually increased from 53.8% in 2009 and reached 100% by 2012. As depicted in Fig. 5, the ptxP3 type was observed in only ST3, ST2, and ST8. Other STs showed the ptxP1 type. The emergence of the ptxP3 type was dependent on the isolation year because only the strains isolated later than 2009 showed the ptxP3 type, even in the same STs. In the case of ST3, the strains in ST3 were divided into three groups according to isolation year (2000, 2009, 2011). From these groups, only the strains isolated in 2011 showed ptxP3 type.

DISCUSSION

A dynamic change of antigenic genotypes in the pertussis pathogen was confirmed in this study. The change commenced in 2009 and continued until 2012. We have to consider two points: the change in the ST of the strains and the emergence of toxic strains showing increased pertussis toxin production due to a mutation in the promoter region (ptxP3 type). As expected, the ptxP3 type was first detected in 2009. The frequency of ptxP3 gradually increased from 53.8% in 2009 and reached 100% by 2012. As depicted in Fig. 5, the ptxP3 type was observed in only ST3, ST2, and ST8. Other STs showed the ptxP1 type. The emergence of the ptxP3 type was dependent on the isolation year because only the strains isolated later than 2009 showed the ptxP3 type, even in the same STs. In the case of ST3, the strains in ST3 were divided into three groups according to isolation year (2000, 2009, 2011). From these groups, only the strains isolated in 2011 showed ptxP3 type.

As described, there were reports (12, 13) of more toxic strains showing increased pertussis toxin production due to a mutation in the promoter region (ptxP3 type). As expected, the ptxP3 type was first detected in 2009. The frequency of ptxP3 gradually increased from 53.8% in 2009 and reached 100% by 2012. As depicted in Fig. 5, the ptxP3 type was observed in only ST3, ST2, and ST8. Other STs showed the ptxP1 type. The emergence of the ptxP3 type was dependent on the isolation year because only the strains isolated later than 2009 showed the ptxP3 type, even in the same STs. In the case of ST3, the strains in ST3 were divided into three groups according to isolation year (2000, 2009, 2011). From these groups, only the strains isolated in 2011 showed ptxP3 type.

Fig. 4. Minimum spanning tree by MLST profiles according to the isolation year. A minimum spanning tree was reconstituted according to the isolation year. The increase in the number of pertussis cases (Fig. 1) is associated with expansion of the ACC I-ST3 subgroup since 2009. In particular, the ST3-ST2-ST8 lineage is expanded in the increasing stage (2009-2011). Compared with 2009 isolates, there are few isolates belonging to the ACC II-ST1 subgroup.

Fig. 5. Minimum spanning tree by MLST profiles according to the variant type of pertussis toxin promoter. A minimum spanning tree was reconstituted according to the variant type of the pertussis toxin promoter. The pertussis toxin promoter (ptxP) 3 type is observed only in 3 sequence types (ST3, ST2, and ST8). The presence of the ptxP3 type might be dependent on the isolation year because ST2 and ST8 have recently expanded.
ptxP3 type in the 2009 isolates was 58.3%, and this increased to 86.6% in the 2011 isolates. Finally, all isolates of 2012 showed the ptxP3 type. Therefore, we conclude that the recently observed increase in pertussis cases in Korea is closely related to the emergence of variant strains.

Global trends reported for genetic variations were similar to our results, except for fimbriae (19). The genotypes of ptxS1, prn, and ptxP3 were similar to global trends. However, the fimbriae genotypes were different. The major genotypes of fim2 and fim3 according to global trends changed from fim2-1 to fim2-2 and from fim3-1 to fim3-2. In Korea, the fim2 genotype did not change (fim2-1), but the fim3 genotype changed from fim3-2 to fim3-1. These results indicate that the Korean strains are changing from the vaccine type in common with the global strain.

The acquisition mechanism of these variations is not clearly resolved. However, it was inferred that the insertion sequence element (IS481) has an important role in the adaptation of B. pertussis to its host (19, 21). The genetic change mediated by IS481 is a large-scale chromosomal rearrangement. The genome reduction and gene loss are caused by this genetic event. According to one report, the genome size of current strains has been gradually decreasing since 1950 (21). Other genetic changes that have been recorded are In/Del polymorphisms in repeat units and single nucleotide polymorphisms (SNPs). The representative case for In/Del polymorphism was observed in the pertactin structural gene (20, 21). As described above, the variants in the prn gene are the result of changes in the number of short tandem repeat units and it was caused by homologous recombination. Another form of genetic changes frequently observed in the B. pertussis genome is SNPs (silent and non-silent SNPs). Especially, non-silent SNPs found in surface antigens cause structural changes in the epitope region and may affect the immune response (19, 21). In addition, SNPs affect regulatory elements of genes related to virulence. The representative case was the promoter variants of the pertussis toxin gene. Currently, 14 variants types are known and the variation was observed in the BvgA binding site, the global regulator of virulence gene expression. Among these variants, the ptxP3 type shows high expression (more than 2-fold) of the pertussis toxin (13) and has an effect on colonization in respiratory tract (22).

As noted above, there have been many reports of emerging genetic variants of B. pertussis strains since 1990. The remaining question from these reports is that the observed genotype polymorphisms were attributed in simple drift event or evolutionary event (6). Currently, it might be concluded as evolutionary event by active adaptation of the pathogen to avoid massive vaccination effects (19). Therefore, it is more important to predict the direction of genotype changes in current strains to cope with emerging new toxic variants. For this reason, the study of genotype variation is gradually moving toward comparative genomic studies to obtain extensive data occurred in whole genome (21). Although MLST analysis for bacterial typing allows rapid generation of clear results and easy comparisons with results (23), the data obtained from MLST analysis are restricted to only the selected gene group and are more suitable for a retrospective study. In addition, the discriminating power is less than that of other typing methods, such as pulsed-field gel electrophoresis (PFGE) or multiple locus variable-number tandem repeat analysis (MLVA) (24). PFGE is known as the gold standard for bacterial typing but it is too laborious and difficult to standardize (25). Therefore, PCR-based typing methods, such as MLVA and MLST, are used more frequently because these methods are relatively simple and easy. In addition, the discriminating power can be increased PFGE level, if MLVA data are combined with MLST data (25). Taken the above into consideration, the genotype variations of the Korean strains confirmed in this study are insufficient to predict future trends in emerging of toxic strain. Therefore, a comparative genomic study is needed between isolated Korean strains.

In conclusion, the present study confirmed that the change in the strains in Korea is an ongoing event. It cannot be predicted whether the ST2 strain will continue to be a major ST or whether other ST strains carrying the ptxP3 gene will emerge. Therefore, the current surveillance system for pertussis should be maintained and genetic characterization of the strains according to their STs should be extended to the whole genome sequence level.

DISCLOSURE

The authors have no conflicts of interest to disclose.

REFERENCES

1. Mattoo S, Cherry JD. Molecular pathogenesis, epidemiology, and clinical manifestations of respiratory infections due to Bordetella pertussis and other Bordetella subspecies. Clin Microbiol Rev 2005; 18: 326-82.
2. De Melker HE, Schellekens JE, Neppelenbroek SE, Mooi FR, Rümke HC, Coyn-van Spaendonck MA. Reemergence of pertussis in the highly vaccinated population of the Netherlands: observations on surveillance data. Emerg Infect Dis 2000; 6: 348-57.
3. World Health Organization. Pertussis vaccines: WHO position paper. Wkly Epidemiol Rec 2005; 80: 31-9.
4. Rothstein E, Edwards K. Health burden of pertussis in adolescents and adults. Pediatr Infect Dis J 2005; 24: S44-7.
5. Hochwald O, Bamberger E, Srugo I. The return of pertussis: who is responsible? what can be done? Isr Med Assoc J 2006; 8: 301-7.
6. Mooi FR, van Loo IH, King AJ. Adaptation of Bordetella pertussis to vaccination: a cause for its reemergence? Emerg Infect Dis 2001; 7: 526-8.
7. Hallander HO, Advani A, Donnelly D, Gustafsson L, Carlsson RM. Shifts of Bordetella pertussis variants in Sweden from 1970 to 2003, during three periods marked by different vaccination programs. J Clin Microbiol 2005; 43: 2856-65.
8. Elomaa A, Advani A, Donnelly D, Antila M, Mertsola J, Hallander H, He Q. Strain variation among Bordetella pertussis isolates in Finland, where the whole-cell pertussis vaccine has been used for 50 years. J Clin Microbiol 2005; 43: 3681-7.
9. Centers for Disease Control and Prevention (CDC). FDA approval of expanded age indication for a tetanus toxoid, reduced diphtheria toxoid and acellular pertussis vaccine. MMWR Morb Mortal Wkly Rep 2009; 58: 374-5.
10. Centers for Disease Control and Prevention (CDC). FDA approval of expanded age indication for a tetanus toxoid, reduced diphtheria toxoid and acellular pertussis vaccine. MMWR Morb Mortal Wkly Rep 2011; 60: 1279-80.
11. Van Loo IH, Heuvelman KJ, King AJ, Mooi FR. Multilocus sequence typing of Bordetella pertussis based on surface protein genes. J Clin Microbiol 2002; 40: 1994-2001.
12. Packard ER, Parton R, Coote JG, Fry NK. Sequence variation and conservation in virulence-related genes of Bordetella pertussis isolates from the UK. J Med Microbiol 2004; 53: 355-65.
13. Mooi FR, van Loo IH, van Gent M, He Q, Bart MJ, Heuvelman KJ, de Greeff SC, Diavatopoulos D, Teunis P, Nagelkerke N, et al. Bordetella pertussis strains with increased toxin production associated with pertussis resurgence. Emerg Infect Dis 2009; 15: 1206-13.
14. Jung SO, Moon YM, Kim SH, Sung HY, Kwon SJ, Kang YH, Yu JY. Multilocus sequence analysis of housekeeping genes and antigenic determinant genes in Bordetella pertussis strains isolated in Korea. Osong Public Health Res Perspect 2011; 2: 115-26.
15. Kumar S, Tamura K, Nei M. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform 2004; 5: 150-63.
16. Jolley KA, Feil EJ, Chan MS, Maiden MC. Sequence type analysis and recombinational tests (START). Bioinformatics 2001; 17: 1230-1.
17. Francisco AP, Vaz C, Monteiro PT, Melo-Cristino J, Ramirez M, Carriço JA. PHYLVIZ: phylogenetic inference and data visualization for sequence based typing methods. BMC Bioinformatics 2012; 13: 87.
18. Diavatopoulos DA, Cummings CA, Schouls LM, Brinig MM, Relman DA, Mooi FR. Bordetella pertussis, the causative agent of whooping cough, evolved from a distinct, human-associated lineage of B. bronchiseptica. PLoS Pathog 2005; 1: e45.
19. Mooi FR. Bordetella pertussis and vaccination: the persistence of a genetically monomorphic pathogen. Infect Genet Evol 2010; 10: 36-49.
20. Mooi FR, van Oirschot H, Heuvelman K, van der Heide HG, Gaastra W, Willems RJ. Polymorphism in the Bordetella pertussis virulence factors P69/pertactin and pertussis toxin in the Netherlands: temporal trends and evidence for vaccine-driven evolution. Infect Immun 1998; 66: 670-5.
21. King AJ, van Gorkom T, van der Heide HG, Advani A, van der Lee S. Changes in the genomic content of circulating Bordetella pertussis strains isolated from the Netherlands, Sweden, Japan and Australia: adaptive evolution or drift? BMC Genomics 2010; 11: 64.
22. King AJ, van der Lee S, Mohangoo A, van Gent M, van der Ark A, van de Waterbeemd B. Genome-wide gene expression analysis of Bordetella pertussis isolates associated with a resurgence in pertussis: elucidation of factors involved in the increased fitness of epidemic strains. PLoS One 2013; 8: e66150.
23. Spratt BG. Multilocus sequence typing: molecular typing of bacterial pathogens in an era of rapid DNA sequencing and the internet. Curr Opin Microbiol 1999; 2: 312-6.
24. Schmidtke AJ, Boney KO, Martin SW, Skoff TH, Tondella ML, Tatti KM. Population diversity among Bordetella pertussis isolates, United States, 1935-2009. Emerg Infect Dis 2012; 18: 1248-55.
25. Advani A, van der Heide HG, Hallander HO, Mooi FR. Analysis of Swedish Bordetella pertussis isolates with three typing methods: characterization of an epidemic lineage. J Microbiol Methods 2009; 78: 297-301.