REPORT

Shattering a myth: Whooping cough susceptible to antibiotics

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Abstract: *Bordetella parapertussis* is the causative agent of a milder form of pertussis or whooping cough. Little is reported about the antibiotic resistance patterns and mechanism of drug resistance of *Bordetella parapertussis*. The objective of this study has been to investigate antimicrobial resistance, distribution of integrons and presence of gene cassettes to quinolones (*qnr*) and sulfonamides (*sul*) among *B. parapertussis* strains' isolated from Pakistan. Thirty-five (35) samples were collected from various hospitals of Pakistan from children (median age 3 years) with pertussis-like symptoms, all were tested and confirmed to be *B. Parapertussis.* Resistance profile of Ampicillin, Cephalexin, Sulphamethoxazole, Chloramphenicol, Ofloxacin, Nalidixic acid, Gentamycin and Erythromycin were investigated through all samples. Majority of the isolates were found to be resistant to the afore-mentioned antibiotics except erythromycin. All isolates were resistant to quinolones phenotypically, but *qnr* genes were detected in only 25.7% (9/35) of isolates. On the other hand, 71.4% (25/35) isolates were resistant to sulfonamides phenotypically. From these 71% strains showing phenotypical resistance, 96% (24/25) were found to possess *sul* genes. Only two isolates were carrying class 1 integrons, which also harbored *sul* gene and qnr gene cassettes. It can be safely concluded that the phenotypic resistance patterns seemed mostly independent of presence of integrons. However, interestingly both integrons harboring strains were resistant to quinolones and sulfonamides and also possessed *qnr* and *sul* genes.

Keywords: *B. parapertussis*; drug resistance; integrons; quinolones; sulfonamides.

INTRODUCTION

Pertussis or whooping cough is an acute respiratory tract disease, caused by bacterial species *Bordetella pertussis* and *Bordetella parapertussis.* It is highly communicable that transmits through airborne droplets. The causative agent is highly infectious and can easily transmit from person to person if precautionary measures are not taken. The children under five are mostly prone to this infection, but whooping cough is not just a childhood disease. It has been found extremely inimical for neonates and infants though can be very severe for children and adults too. Nonetheless, the incidence of disease has been greatly reduced because of infant vaccination programmes (Liese *et al*., 2003; Mattoo and Cherry, 2005). Vaccination against *B. pertussis* is available in two forms, whole cell pertussis vaccine (DTwP) and acellular pertussis vaccine (DTaP). These are usually given in combination with diphtheria and tetanus toxoids adsorbed on aluminium salts. As DTaP is associated with less side effect, so is replacing DTwP (Guiso *et al*., 2001; Njamkepo *et al*., 2002). Both vaccines are effective only against *B. pertussis*, which causes severe whooping cough. However, *B. parapertussis* causes the milder form of whooping cough and vaccination is not effective against *B. parapertussis*.

B. parapertussis infections are common in both developing (Bokhari *et al*., 2011) and developed countries (Njamkepo *et al*., 2002). *B. parapertussis* are considered to be susceptible to most of the commonly used antibiotics (Kurzynsky *et al*., 1988; Tiwari *et al.*, 2005). However, the macrolide antibiotic such as erythromycin is considered a drug of choice for the treatment of pertussis, whereas an alternative option is sulfamethoxazole-trimethoprim (Tiwari *et al.*, 2005). Increase in bacterial resistance to different classes of drugs is being reported in many countries of the world. However, the situation seems quite worse in third world countries including Pakistan due to injudicious use of antibiotics (Moreno *et al.*, 2006; Kim *et al*., 2009; Sapiro, 2002; Idrees *et al*., 2011; Gour and English, 2006). Quinolones and sulfonamides are also commonly used antibiotics to treat bacterial infections including *Bordetella.*

Previous studies suggest that the *sul* gene is associated with sulfonamide resistance phenotype (Skoed, 2000; Antunes *et al*., 2005). Three types of *sul* genes (*i.e. sul1*, *sul2* and *sul3*) have so far been reported (Antunes *et al*., 2005). Bacterial resistance to Quinolones has traditionally been attributed to chromosomal mutations that alter quinolones targets (DNA gyrase and topoisomerase IV) and over production of efflux pumps. Three classes of plasmid mediated quinolone resistance genes (*qnrA*, *qnrB* and *qnrS*) have been identified encoding for pentapeptide family of proteins, which inhibit quinolone binding to DNA gyrase and topoisomerase IV (Li, 2005).

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Dissemination of antibiotic resistance genes by horizontal gene transfer has led to rapid emergence of antibiotic resistance in clinical isolates. In this regard, integrons play a significant role in the mobilization of antibiotic resistance genes among bacterial pathogens (Vo *et al*., 2010). Four classes of integrons have so far been identified and classified on the basis of their integrase type. Class 1 Integrons are the most common type of integrons found in many bacterial species (Gillings *et al*., 2008; Kadlec *et al.*, 2005).

Although much of researches has been published on antibiotic resistance but still a reliable surveillance data still lacks in many countries of the world including Pakistan. There are very few studies that focus *B. pertussis* and *B. parapertussis* particularly in Pakistan. In this study, effort will be made to relate phenotypic drug resistance (by disc diffusion method) to already reported genotype and focus would be on quinolone (*qnr*) and sulfonamide (*sul*) resistance genes. Furthermore, these isolates would be screened for the possible presence of integrons.

MATERIAL AND METHODS

Ethical considerations

The prior approval from the Departmental Ethical Committee was obtained. A written consent was taken from all the participants. The medical history of the participants was obtained with the help of a questionnaire.

Sample collection

Samples were collected from suspected pertussis patients from various hospitals of Pakistan by nasopharyngeal swabs (NPS). All samples were transported to laboratory within four hours of collection.

Isolation and characterization of B. parapertussis strains

Bordet Gengou agar (BG agar) supplemented with 10-15% sheep blood was used to cultivate bacteria. NPS were streaked onto fresh BG agar supplemented with sheep blood. Plates were then incubated for seven days at 37^{o}C. Plates were inspected on every alternate day for noticing any growth. If typical growth was found, it was transferred on fresh BG agar plate and further biochemical tests were done to identify microbes. Bacterial identification was done by Gram-staining and by biochemical tests.

Antibiotic sensitivity testing by disc diffusion method

Bordet Gengou agar (Oxoid, UK) supplemented with 10-15% sheep blood was used to study the antibacterial sensitivity testing by using disc diffusion method for a number of antibiotics (fig. 1). Strains showing resistance to trimethoprim-sulfamethoxazole and quinolones (nalidixic acid and ofloxacin) were shortlisted for the PCR detection of quinolone (*qnr*) and sulfonamide (*sul*) resistance genes.

DNA extraction

DNA was extracted from bacterial cells following a modified form of phenol chloroform method described by Chang and Jiang (2006). Bacterial colonies were picked from the agar plates with the help of a wire loop and suspended in 1ml phosphate buffered saline (PBS). It was then centrifuged at 8,000g for 2 minutes. After removing the supernatant, pelleted cells were washed twice with 400µL STE buffer (100mM NaCl, 10mM Tris-HCl and 1mM EDTA, pH 8). The cells were centrifuged at 8000g for 2 minutes. The cell pellet was re-suspended into 200µL of TE buffer (Tris-HCl=10mM, EDTA=1mM, pH 8.0). 100µL of Tris saturated phenol was added to this tube followed by vortexing for 1.0 minute to lyse the cells. Samples were then centrifuged at 13,000g for 5 minutes at 4°C to separate the aqueous phase from organic phase. Next, 160µL of upper aqueous phase was transferred into a new tube. TE buffer (40µL) was added to make it to 200µL. 100µL of chloroform was added to the sample followed by the centrifugation at 13,000g for 5 minutes. 160µL supernatant was taken into another tube and the 40µL of TE buffer was added and centrifuged at 13,000g for 5 minutes. Extraction with chloroform step was repeated twice. The supernatant containing DNA was taken into a clean sterilized tube and stored at -20°C for long term or 4-8°C for short-term storage.

Polymerase chain reaction (PCR) for bacterial identification

A number of previously described PCR primers were used for identification of *Bordetella* isolates. *IS1002* is an insertion sequence present in both *B. pertussis* and *B. parapertussis* (Van der Zee *et al*., 1996). These primers served for initial screening of the isolates for presence of either of the species, whereas, *IS1001* is specific for detection of *B. parapertussis* (Van der Zee *et al.*, 1993). Primers were diluted to a concentration of about 0.1 µMol for stock (10X) and refrigerated until used. All primers used in the study were purchased from a commercial source (Alpha DNA, Canada).

PCR detection of quinolone and sulfamethoxazole resistance gene

Resistance genes were detected by PCR using various primers (table 1) for different types of *qnr* (*qnrA, qnrB* and *qnrS*) and *sul* (*sul1 sul2* and *sul3*) genes. All primers were bought from a commercial source (Alpha DNA, Canada).

PCR detection of integrons and their typing

PCR detection of integrons was carried out by using primers Hep 35 and Hep 36 encoding for integron associated integrase (White *et al.*, 2001). The 491 bp product was digested with *hinf1* (Fermentas, USA)

restriction enzyme for classification of Integrons into class 1, 2 and 3.

Phylogenetic analysis of integrase gene segment

A PCR amplified products (using Hep35 & Hep36 primers) obtained by targeting Integron associated integrase gene from two *B. paraperussis* were gel extracted from low melting agarose (2%) using Qiagen gel extraction kit. The samples were sequenced (Macrogen, Korea) in both directions. Sequence alignment was performed with the multiple sequence alignment software CLUSTAL X ver. 1.83. These alignments were subjected to phylogenetic neighbour joining analysis. For neighbour joining, distance matrices were computed through Protdist component of Phylip software suite. To confirm neighbour joining results the alignments were also subjected to Bayesian analysis using MrBayes. Trees were drawn using TreeDyn.

RESULTS

Strain characterization

All thirty-five strains showed growth after 2-3 days on BG media. All strains were Gram negative. All were oxidase negative and urease positive. These results indicate the growth of *B. parapertussis:* However, further confirmation was done by PCR.

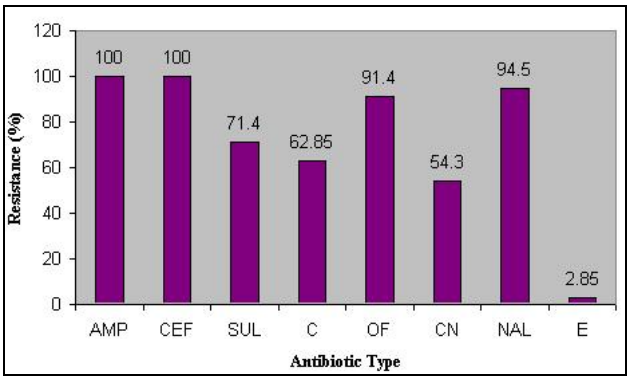

Fig. 1: Antibiotic resistance patterns of *B. parapertussis* strains against different antibiotics. Abbreviations: AMP= Ampicillin, CEF=Cephalexin, SUL= Sulfamethoxazole, C= Chloramphenicol, OF= Ofloxacin, CN= Gentamycin, NAL= Nalidixic acid, E= Erythromycin

Study of antibiotic resistance patterns by disc diffusion method

Antibiotic sensitivity testing was carried out against a number of antibiotics. Majority of the strains were found to be resistant to the antibiotics earlier discussed except erythromycin. Details of antibiotic resistance patterns are given in fig. 1. Only those strains that were showing phenotypic resistance (by zones of inhibition) for quinolones (Ofloxacin or Nalidixic acid) and sulphonamides were shortlisted for studying genotype.

PCR detection of quinolone resistance gene

Resistance genes were only detected for quinolones and sulfonamides. All thirty five isolates were found to be resistant to quinolones phenotypically either to Nalidixic acid or Ofloxacin as observed in fig.1, but *qnr* genes were detected in only 9 isolates (fig. 2, table 2) i.e., in (25.71%) of the samples. The proportion of different types of *qnr* genes for *qnrA was* 44.4% (n=4), *qnrB* was 0% (n=0) and *qnrS* was 55.6% (n=5). Only a single sample had two *qnr* genes (*i.e. qnrA* and *qnrS*).

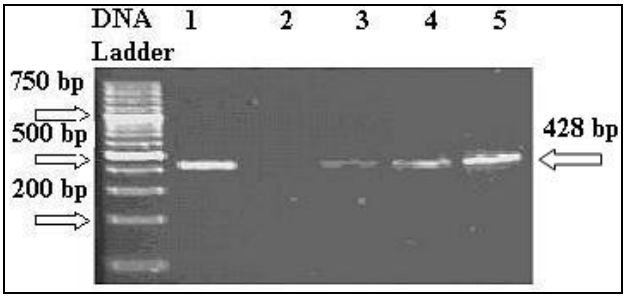

Fig. 2: Agarose gel electrophoresis of PCR amplification product of *qnrS* gene segment (Lanes 3-5). Lanes 1 and 2 are positive and negative controls respectively.

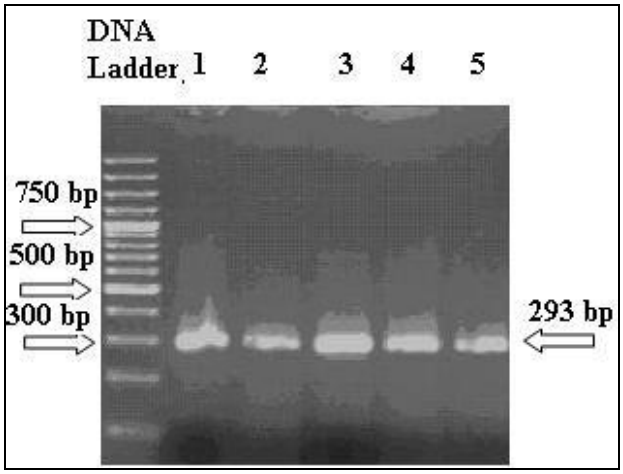

Fig. 3: Agarose gel electrophoresis of PCR amplification products of *sul2* gene segment (Lanes 1-5).

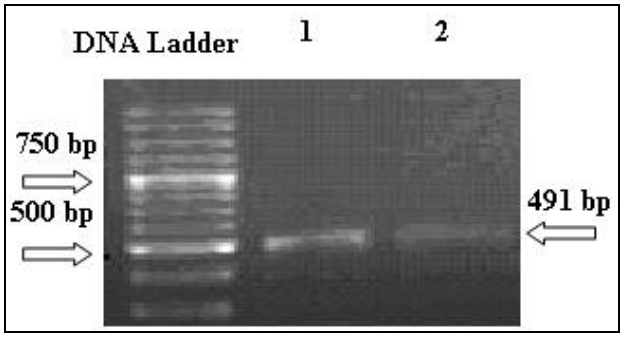

Fig. 4: Agarose gel electrophoresis of PCR amplification product of integron sequence.

PCR detection of sulfonamide resistance genes

25 strains were found to be phenotypically resistant to sulfamethoxazole disc. So (71.4%) displayed resistance

Table 1 List of all primers used in the study for the identification of *B. parapertussis* strains for antibiotic resistance genes and integron detection

Gene/target	Primer	Sequence	Product size (bp)
IS1001	BPPA BPPZ	5`-CGCCGCTTGATGACCTTGATA-3` `5-CACCGCCTACGAGTTGGAGAT-3`	498
IS1002	GH1 HG2	5'-GCCGATGCGTTCCATATA-3` 5`-AGCCCTTCTTGTTAACAGGG-3`	293
Integrase	Hep35 Hep36	5`-TGCGGGTYAARGATBTKGATTT-3` 5`-CARCACATGCGTRTARAT-3`	491
sul1	sul1-F sul1- R	5`-CGGCGTGGGCTACCTGAACG-3` 5`-GCCGATCGCGTGAAGTTCCG-3`	433
sul2	sul2 -F sul2 -R	5`-GCGCTCAAGGCAGATGGCATT-3` 5`-GCGTTTGATACCGGCACCCGT-3`	293
sul3	sul3-F sul3-R	5`GAGCAAGATTTTTGGAATCG-3` 5`-CATCTGCAGCTAACCTAGGGCTTTGGA-3`	772
qnrA	qnrAm-F qnrAm-R	5`-AGAGGATTTCTCACGCCAGG-3` 5`-TGCCAGGCACAGATCTTGAC-3`	580
qnrB	qnrBm-F qnrBm-R	5`-GGMATHGAAATTCGCCACTG-3` 5`-TTTGCYGYYCGCCAGTCGAA-3`	264
qnrS	qnrSm-F qnrSm-R	5`-GCAAGTTCATTGAACAGGGT-3` 5`-TCTAAACCGTCGAGTTCGGCG-3`	428

Table 2 Proportion of different types of sulfonamide (*sul*) and quinolone (*qnr*) resistance genes in *B. parapertussis* strains isolated from Pakistan

S No.	Sulfamethoxazole Resistance gene (s)	Proportion (n/24)	Qunilone resistance gene (s)	Proportion (n/35)
1	Sul 1	75% (18)	qnrA	44.4% (4)
2	Sul 2	58.3% (14)	qnrB	0% (0)
3	Sul 3	8.3% (2)	qnrS	55.6% (5)
4	Sul 1+sul2	33.3% (8)	qnrA+qnrB	0% (0)
5	Sul 2+su3	0 (0)	qnrA+qnrS	11.1% (1)
6	Sul 1+sul3	8.3% (2)	qnrB+qnrS	0% (0)
7	Sul 1+sul2+sul3	0% (0)	qnrA+qnrB+ qnrS	0% (0)

for solfonamides by studying zones of inhibition. All these resistant samples were subjected to PCR to detect the presence of *sul* genes. From these 25 samples, 24 samples i.e., 96% contained at least one type of *sul* genes (fig. 3, table 2).

The proportion of *sul1, sul2* and *sul3* genes was 75% (n=18), 58.3% (n=14) and % 8.3 (n=2) respectively. The co-occurrence of two *sul* types *i.e., sul1 + sul2* and *sul1 + sul3* have been seen in 33.3% (n=8) and 8.3% (n=2) of isolates respectively, whereas none of the isolate possessed all three genes (*i.e. sul1, sul2 and sul3*) together.

Detection of integrons in the B. parapertussis strains

The study showed the presence of integron in two of the *B. parapertussis* strains (fig. 4). One strain was from west of the country (Sindh province), while the other was from KPk (Khyber Pakhtoonkhwa province). RFLP analysis with *Hinf1* identified it as class 1 integron, due to lack of restriction. Sequence analysis using BLAST showed similar integron1 sequences (up to 99% homology) present in a number of bacterial species. Interestingly, both integron bearing strains were also resistant to quinolones and sulfonamides. *sul1* and *sul2* were present in both integron harbouring strains whereas only a single type of *qnr* genes (i.e. *qnrS*) was detected in these two strains.

DISCUSSION

As pertussis is an underestimated disease that often goes unnoticed unrecognized by physicians (Syed *et al*., 2009; Syed and Bukhari, 2010) a reliable surveillance data still lacks in many countries of the world including Pakistan (Bokhari *et al*., 2011). Nonetheless, high prevalence of both *B. pertussis* and *B. parapertussis* infections is suspected in people of all age groups (Mattoo and Cherry, 2005). In this study, an attempt was made to study the antibiotic resistance to a number of antibiotics phenotypically and of quinolones and sulfonamides genotypically by detecting *qnr* and *sul* genes, responsible for resistance to these antibiotics.

Both *B. pertussis* and *B. parapertussis* are generally considered to be susceptible to commonly used antibiotics. Diagnostic laboratories routinely do not perform susceptibility testing for these species (Mattoo and Cherry, 2005). In contrast to the previous report (Kurzynsky *et al.*, 1988), results of this study report a high level of resistance to both quinolones and sulfonamides.

Both chromosomal and plasmid mediated quinolone resistance has been observed in different bacterial species as reported previously (Li, 2005). In this study, almost all strains showed resistance to quinolones phenotypically (either to Nalidixic acid or Ofloxacin), but *qnr* genes were detected in only 25%. Therefore, it is concluded that main mechanism of quinolone resistance is independent of *qnr* in *B. parapertussis* and may be chromosomally controlled or perhaps controlled by some other plasmid borne gene.

Sulphonamide resistance was observed phenotypically in 71% of samples and *sul* gene was detected by PCR in 96% of these samples. This indicates a strong relationship of sulphonamide phenotypic drug resistance with *sul* gene. Proportion of *sul1* and *sul2* genes was higher as compared to *sul3* in these strains, which coincides with the already reported incidences in other bacterial species (Hassan *et al*., 2008).

In this study, presence of class 1 integrons in two of the isolates was observed. Presence of integrons has already been reported in *B. bronchiseptica* (Kadlec *et al*., 2005) and this paper is the first report on their occurrence in *B. parapertussis*. Both isolates carrying integrons were from two different provinces well apart from each other. Moreover, BLAST and phylogenetic analysis of the integron sequences showed that similar sequences are also present in a number of bacterial species, which may indicate possible horizontal gene transfer of these elements.

High level of drug resistance and presence of resistance genes in *B. parapertussis* is a matter of concern in this part of the world, as results of this study contrast the previous studies showing susceptibility of this organism to most of the useful antibiotics (Kurzynski *et al.*, 1988). Overuse of antibacterial drugs is a key factor in an overall rise in the drug resistance in the developing countries including Pakistan (Hassan *et al*., 2008; Cattoir *et al.*, 2007; Idrees *et al.*, 2011). In the course of research it was proved that (i) there was high resistance to different classes of antibiotics (ii) quinolone and sulphonamide resistance was significantly higher compared to other antibiotics (iii) the presence of class 1 integrons was also seen in two *B. parapertussis* isolates (iv) and finally, analysis of *B. parapertussis* isolates suggested the existence of resistance to quinolones and sulphonamides was independent of integrons in most of the cases.

CONCLUSIONS

This is the first report on the antimicrobial sensitivity testing of *B. parapertussis* strains from Pakistan. Its results are interesting, as all the patients with whooping cough like symptoms were found to be positive for *B. parapertussis* only. *Bordetella* infections are generally considered to be susceptible to commonly used antibiotics, however, results of this study have shattered the general perception. An unusual higher level of antibiotic resistance was observed both phenotypically and genotypically. This study also reports presence of Type 1 integrons in *B. parapertussis*, which is first to the knowledge of the researchers. There is an urgent need of surveillance of *Bordetella* infections in Pakistan on a broader scale.

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