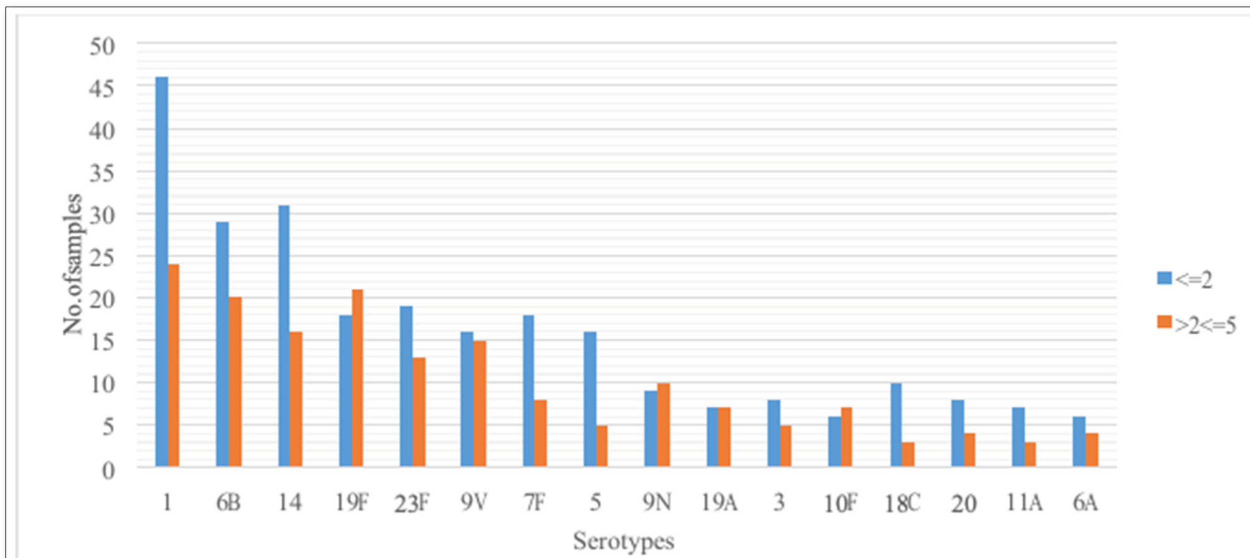

Figure 3. Age wise distribution of serotypes.

DISCUSSION

Isolation of S. pneumoniae from a normally sterile body site provides conclusive evidence of invasive pneumococcal infection, but this is achieved only for a minority of cases [10]. Despite the global importance of pneumococcal disease, there have been surprisingly few recent developments in laboratory diagnostics. [10,11] The capacity to obtain accurate data on IPD burden and to assess the effectiveness of vaccination is hindered by the limitations of existing diagnostic tests. To conquer these limitations, we developed and evaluated a highly sensitive and specific quantitative multiplex real time PCR assay for the identification of S. pneumoniae from culture positive and culture negative samples.

Several molecular PCR typing protocols for S. pneumoniae are described targeting serotype specific regions of cps. [9,12-16]. Nevertheless, these methods were of low resolution between cross reactive serotypes and differentiation of cross-reactive serotypes required a high number of primers. Methods for rapid and reliable serotyping of Streptococcus pneumoniae not based on bacterial culture are needed in the epidemiological surveillance of pneumococcal infection. The development of PCR-based serotyping assays performed directly on clinical samples that are not required to contain viable bacteria has the potential to overcome some of the difficulties associated with these microbiological diagnostic procedures that can perform poorly, especially in pediatric patients [16]. We have developed a PCR and sequencing-based assay to identify all known and emerging serotypes from clinical isolates. In the present study, we report the performance of qmPCR and PCRSeqTyping assay directly on clinical samples from culture negative patients.

Serotype distribution of the pneumococcus changes with time due to various factors including clonal enlargement, capsular transformation, mass and routine pneumococcal vaccination, and widespread antibiotic use in population. Pneumococcal serotype appears to be much more important in determining colonization, disease development, and clinical phenotype compared to the genetic background [17]. Thus, obtaining information about local serotype distribution of pneumococci and observing potential changes in the distribution in time is essential for effective vaccination strategies. The most common serotypes causing IPD in European children are 14, 6B, 19F and 23F, which are included in PCV7 [18]. A recent global review on pneumococcal serotypes causing invasive disease in 51 countries showed that serotype 14 was the most common in all regions including Africa, Asia, Europe, Latin America, North America and Oceania [19]. They observed that seven serotypes 1, 5, 6A, 6B, 14, 19F and 23F accounted for 66-76% of diseases in each region, and the top eight serotypes in Africa and Asia were the same which included, the seven serotypes mentioned in addition to serotype 19A. It was also observed that serotype 14 was the dominant serotype in younger children (<23 months), while serotype 1 was the dominant serotype in older children (24-59 months).

Estimating the true pneumococcal disease burden in Indian continues to be challenging, while morbidity and mortality rates remain high [20]. Hence, studies on serotype prevalence are still relevant in the Indian context. There is a paucity in data on serotypes from India as some of the

studies conducted had limited sample size and failed to contribute to epidemiological analysis [21]. Some of the studies were conducted almost a decade back during which the epidemiology of S. pneumoniae might have changed to a great extent [22].

The analysis of Indian publications since 1999 related to invasive pneumococcal disease serotypes revealed serotype 1 as the most common followed by 6A, 19F, 14, 6B and 5. Studies from South India, have reported 1, 6A, 14, 5 as the most common serotypes **(Table 3)**. A multicenter hospital surveillance study reported 6, 1, 19 as the most prevalent serotypes among children <5yrs of age. This emphasizes the geographic variability of pneumococcal serotypes in a vast country like India. A combined analysis of serotype distribution among South Asian Association for Regional Cooperation nations by Jaiswal and colleagues [22], has shown the prevalence of serotypes 1, 5, 14, 12A, 12F, 7F, 15B, 4, 19F, 19A, 6B, 23F, 2, 6A, 9V and 18C in Nepal, Bangladesh, Sri Lanka and India.

Table 3. India Historic data: Pneumococcal studies from 2000-2019.

Study	Study Year/Publication year	Place	Sample no	Isolates	Serogroup or types	Reference
Mahajan [23]	1987	New Delhi	--	171, Ocular infections	3,46,8,28,29, 45, 21, 18	28
John [24]	1996	Vellore	NA	42	1,5,7	29
IBIS study [25]	1997	Vellore	5798	307	1,5,19	30
Lalitha [26]	1997	Vellore	NA	150	1,6,7,15,5	31
Cloes [27]	1999	Madurai	384	30	6,14,15	32
Rajalakshmi [28]	2001	Pondicherry	150	109	1,6,5,19	33
Kanungo [29]	2001	Pondicherry	NA	124	1, 6, 19, 5, 23 and 7.	34
SAPNA study [30]	2008	Chennai	1791	23	5,14,23F	35
ANSORP study [31]	2009	Indian data	2184	23	19F,5,19A	36
Sherrif [32]	2010	New Delhi	NA	108	7F,1,19A	37
Nisarga [33]	2011	Bangalore	10000	40	6,14,5	38
Molander [34]	2011	Vellore	NA	244	1,5,19F,6B,14	39
Manoharan [35]	2013	Vellore	3572	225	1,5,19F/6B	40
Balaji [36]	2015	Vellore	NA	114	14, 19F, 5, 6A, 6B	41
Manoharan [35]	2017	Multi-enter	4377	361	14,1,5,19F	42
Jayaraman [37]	2018	Vellore	-	408	1,3,5,19F,8,14	43
This Study		Multi-center	1504	108	1,6B, 14, 19F, 23F	

With the characterization of the cps locus of 92 serotypes [38], Leung [8], have recognized a region unique in sequence to all serotypes that is flanked by conserved primer binding sites. Recently diverse groups [5,39,40] have published their results using sequetyping assay as described by Leung [8]. The limitations of Sequetyping method were addressed in the present study.

This study evaluated 1504 serum samples from pediatric patients who had been diagnosed with invasive pneumococcal disease (IPD) from January 2013 to December 2015. High positivity rate for qmPCR (~30%) was observed as compared to conventional methods for isolation of pneumococci (7%). The positivity rates were consistent across different centers of India. Further we were able to deduce serotypes of all 456 qmPCR positive serum samples using PCRSeqTyping. Our study demonstrates the dynamics of diverse serotype distribution of pneumococci in clinical specimens.

PCRSeqTyping based serotyping in PCR positive serum samples indicated the existence geographical variations of serotypes in regions of India. Serotype 1 was observed to predominant in northern part of India and the % distribution was reduced down south. Similarly, serotype was predominant in Southern part of India and its % distribution reduced up north. Few serotypes very uniquely represented in some part of the country. Serotype 9V, 7F, 9N, 23F, 3&4 were found uniquely in Delhi, Jodhpur, Bangalore, Chennai and Kanpur region. Overall serotype 1, 6B, 14, 19F and 23F were predominant serotypes reported from serum samples. This study also demonstrates that pneumococcal serotype can be obtained in cases where is culture is negative. Similar studies were conducted where pneumococcal serotypes were deduced with the aid of molecular serotyping methods directly from blood (201), pleural fluids (202), NP specimens (203), CSF (204) and MEF samples (205). These studies adopted either conventional sequential PCR (93) or real time multiplex sequential PCR (206) for deducing the serotypes from culture negative clinical samples. The major limitation of the studies was that the serotypes of the closely related strains were reported as serogroup. PCRSeqTyping protocol, identified the pneumococcal serotypes directly from serum samples and also assigned the closely related serotypes to their respective types without the issues of cross reaction. The development of modified typing method has the potential to overcome some of the difficulties associated with the conventional serologic method and multiplex/sequential PCR based methods.

Advantages and limitations of PCRSeqTyping

The described method has multiple advantages over other previously reported serotyping methods. It involves techniques with a workflow that many microbiology laboratories can easily implement. The high throughput PCRSeqTyping method features good discriminatory power, reproducibility, and portability, making it suitable for epidemiological studies. The assay has the flexibility of incorporating additional primers for the characterization of emerging serotypes. An added advantage of this method is that raw data from experiments can be reanalyzed upon the addition of new entries to the serotyping database. The limitation of the protocol lies in its inability to quantify the serotype abundance and serotype identification of multiple serotypes commonly encountered in NP samples.

CONCLUSION

Invasive pneumococcal infections are underestimated due to lack of sensitivity of conventional blood culture. Serum qmPCR combined with PCRSeqTyping could identify and serotype, pneumococcal infection precisely in culture negative clinical samples, substantially improving the laboratory diagnosis in children with IPD. Use of these methods will help to estimate the true burden of the disease. Extensive use of this methodology will provide true estimate of disease burden and will help in reducing the treatment failures.

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