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Research paper

Virulence profiling of *Shigella flexneri* and emergence of serotype 2b as a highly virulent shigellosis causing strain in Pakistan

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ABSTRACT

Bacillary diarrhea caused by *Shigella flexneri* is mediated by various virulence factors which make it the leading agent of diarrhea in developing countries. Previously, a high prevalence of *S. flexneri*, associated with diarrhea has been reported in Pakistan but no data is available on their virulence profile. The present study reports for the first time analysis of various virulence factors among *S. flexneri* serotypes isolated from clinical (diarrheal stool) and non-clinical (retail raw foods and drinking water) sources. A total of 199 *S. flexneri* (clinical: 155, raw foods: 22, water: 22) belonging to various serotypes were subjected to virulence genes detection and virulence profiling. The most frequent virulence gene was found to be *ipaH* (100%), followed by *sat* (98%), *ial* (71.3%), *set1B* (65.8%) and *set1A* (38.7%). A high level of virulence was detected in serotype 2b as compared to other serotypes as 32.3% of all serotype 2b have the entire set of five virulence genes including *ipaH* (100%), *ial* (100%), *sat* (37.7%), *set1A* (89.3%), and *set1B* (100%). Seven different virulence gene profiles (V1 - V7) were detected and the most frequently observed to be V1 (*ipaH*+, *ial*+, *sat*+, *set1A*+, *set1B*+) followed by V3 (*ipaH*+, *ial*+, *sat*+, *set1B*+). The predominant virulence gene pattern in serotype 2b isolated from clinical and non-clinical samples were V1 and V3. Furthermore, about 32% strains belonging to serotype 2b contain the complete set of five virulence genes isolated from patients with high disease severity. In conclusion, the current finding revealed for the first times that serotype 2b was the most virulent strains in both clinical and non-clinical samples in Pakistan. In addition, the virulence of serotype 2b was well correlated with high disease severity.

1. Introduction

The genus *Shigella* is a facultative enteric pathogen and belongs to the Enterobacteriaceae family that invade and colonize the host colonic epithelium (Kane and Dorman, 2012; Kotloff et al., 2018; Schroeder and Hilbi, 2008) that subsequently leads to various clinical manifestations depending on the strain virulence potential, the working of host immune system and its nutritional status (Cruz et al., 2014; Fan et al., 2017). There are four clinically important species of *Shigella* such as *S. flexneri, S. sonnei, S. dysenteriae* and *S. boydii* (Grimont et al., 2007; Murray et al., 2017). In developing countries, the most prevalent species is *S. flexneri* (Sharma et al., 2009). *Shigella* species are further classified into serotypes based on unique characteristics of O-antigen present on lipopolysaccharides (LPS) in the outer membrane. There are 19 serotypes of *S. flexneri* which includes 1a, 1b, 1d, 2a, 2b, 3a, 3b, 4a, 4av, 4b, 5a, 5b, 6, X, Y, Xv, Yv, 7a, and 7b. Previous studies showed that the frequency of *Shigella* serotypes may vary among different geographical locations. The most frequent serotypes are 2a, 3a, and 1a in developing countries (Livio et al., 2014; Muthuirulandi Sethuvel et al., 2017; Sun et al., 2013; Ye et al., 2010).

Shigella species produce various virulence factors that are involved in their pathogenesis (Suganya et al., 2016). Several associated virulence factors have been identified in *S. flexneri* either encoded by plasmids or chromosomes. Examples of virulence genes are invasion plasmid antigen H (*ipaH*) and invasion associated locus (*ial*) which facilitate *S. flexneri* to establish infection by enhancing cellular penetration and spreading

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(Pazhani et al., 2008). Other important virulence factors are *Shigella* enterotoxin 1 (*set*) and secreted auto-transporter toxin (*sat*) (Sousa et al., 2013). It is therefore essential to study the virulence characteristics of *S. flexneri* strains collected from drinking water and retail raw foods as well as from clinical sources.

Despite the abundance of reports on the mechanism related to virulence factors in *S. flexneri* from Asian countries, there is no data available on the distribution of *S. flexneri* virulence factors and their association with their serotypes in Pakistan. In order to develop an effective control strategy, it is essential to conduct studies in *S. flexneri* in terms of its virulence. Therefore, we analyzed the distribution of selected virulence genes among different *S. flexneri* strains isolated from clinical and non-clinical samples in Pakistan.

2. Methodology

2.1. Bacterial strains

A total of 199 identified *S. flexneri* strains including 155 clinical strains from diarrheal patient's stool (Nisa et al., 2020) and 44 non-clinical strains (drinking water: 22, and retail rawfood: 22) collected from January 2016 to May 2017 in Pakistan (unpublished work). Molecular serotyping were performed for *S. flexneri* isolates using a multiplex PCR assay (Sun et al., 2011). Pre-informed consent was taken from children's parents/guardian for collection of clinical data and stool samples. *S. flexneri* strains were processed for analysis of virulence genes. Shigellosis severity was also scored as severe (Abdominal pain, fever: ≥39 °C, frequency of bloody stools: ≥6 per day, frequency of vomiting: 2–4 episodes per day), moderate (Abdominal pain, fever: 38.5–38.9 °C, frequency of bloody stools: 4–5 per day, frequency of vomiting episodes: 1 per day), mild (Abdominal pain, fever: 37.1–38.4 °C. frequency of watery stools: 1–3 per day).

2.2. Molecular detection of virulence genes

To investigate which virulence genes are present in the various *S. flexneri* strains, a PCR based assay was performed as described previously (Yaghoubi et al., 2017). Briefly, DNA was extracted from fresh overnight culture and subjected to PCR amplification of selected virulence genes (*ipaH, ial, sat, set1A* and *set1B)* using specific primers as described previously (Ruiz et al., 2002; Yaghoubi et al., 2017) and 2× Phire green hot start II PCR master mix (Thermofisher, CA, USA). Gene product was detected on 1.5% agarose gel (Yaghoubi et al., 2017).

2.3. Statistical analysis

Statistical analysis was performed to find out the association of (i) virulence genes among *S. flexneri* serotypes (ii) virulence gene profiles among the different serotypes of *S. flexneri* (iii) association between virulence gene profiles and disease severity by calculating Pearson's X^2 test and OR (binary logistic regression) with 95% CI. *P*-values <0.05 was considered statistically significant. Statistical analysis was calculated using MS Excel and VassarStats (online statistical tool).

3. Results

3.1. Frequency of virulence genes in S. flexneri

A total of 199 *S. flexneri* strains were screened for five virulence genes (*ipaH*, *ial*, *sat, set1A* and *set1B)*. The most prevalent virulence gene was *ipaH* (100%) followed by *sat* (98%), *ial* (71.3%), *set1B* (65.8%), and *set1A* (38.7%) as shown in Table 1.

ipaH gene was detected in all *S. flexneri* isolated from all clinical and non-clinical samples. The frequency of *ial, sat, set1A,* and *set1B* genes were found to be 67.1%, 98.1%, 37.4%, and 63.9% in clinical samples respectively. While the frequency of *ial, sat, set1A,* and *set1B* genes were found to be 86.4%, 97.7%, 43.2%, and 72.7% in non-clinical samples respectively (Table 1). The difference in the frequency of virulence genes among *S. flexneri* isolated from clinical and non-clinical samples was not statistically significant (Table 1).

3.2. Distribution of virulence genes of S. flexneri among various serotypes and sample source

The frequency of *ipaH* among 2b, 1b, 7a, 2a, 1d, and Y was found to be 37.7%, 24.1%, 19.6%, 11.5%, 5%, and 2% respectively. High rate of *ial* gene was observed in 2b (37.7%), followed by 2a (11.4), 1b (11.1%), 7a (9%), and Y (2%). The *ial* gene was not detected in 1d serotype. The most frequent serotype expressing *sat* gene was 2b (37.7%), followed by 1b (24.1%), 7a (19.6%), 2a (11.4%), and 1d (5%). All Y serotypes didn't exhibit *sat* gene. A highest frequency of *set1A* was found in 2b (33.7%), followed by 1d (4.5%). *set1A* gene was not detected in 2a, 7a, and Y serotype. The frequency of *set1B* among 2b, 2a, 7a, 1b, Y, and 1d was found to be 37.68%, 11.5%, 9%, 5%, 5%, 2%, and 0.5% respectively (Table 2).

The frequency of *ipaH* in serotype 2b was observed as 53 (34.2%) and 22 (50%) among clinical and non-clinical samples respectively. The rate of *ipaH* in *S. flexneri* serotype 7a was observed as 38 (24.5%) and 1 (2.3%) among clinical and non-clinical samples respectively. Statistical difference ($p < 0.05$) was observed for 2b and 7a among clinical and non-clinical samples (Table 2). The *ial* genes among serotype 7a were only present in clinical samples 18 (11.6%) and were not observed in isolates from non-clinical samples. *Ial* gene was not detected in serotype 1d isolated from both clinical and non-clinical samples (Table 2). The frequency of *sat* gene in *S. flexneri* serotype 2b was observed as 53 (34.2%) and 22 (50%) among clinical and non-clinical samples respectively (Table 2). The frequency of *sat* genes among serotype 7a was 38 (24.5%) and 1(2.3%) among clinical and non-clinical samples. The *sat* gene was not detected in both serotype Y isolated from clinical and non-clinical samples (Table 2). The *set1A* gene was not detected in serotype 2a, 7a, and Y isolated from clinical and non-clinical samples. The *set1B* genes among serotype 7a were only present in clinical samples 18 (11.6%) and were not observed in isolates from non-clinical samples (Table 2).

3.3. Virulence gene profiles of S. flexneri

In order to be able to classify the various virulence patterns of

Table 1
Distribution of virulence genes among *S. flexneri* isolated from clinical and non-clinical (retail raw food and drinking water) sources.

Virulence genes	Total isolates, n(%)	Clinical isolates, n(%)	Non-clinical isolates, n(%)	Chi square	OR	0.95 Confidence intervals		p-value
						Lower limit	Upper limit	
ipaH	199(100)	155(100)	44(100)	0.35	1.1	0.78	1.7	0.54
ial	142(71.3)	104(67.1)	38(86.4)	0.93	0.81	0.53	1.21	0.33
sat	195(98)	152(98.1)	43(97.7)	0.37	1.13	0.78	1.71	0.53
set1A	77(38.7)	58(37.4)	19(43.2)	0.04	0.93	0.54	1.6	0.82
set1B	131(65.8)	99(63.9)	32(72.7)	0.05	0.94	0.62	1.49	0.81

Note: p value < 0.05 was considered statistically significant. Non clinical samples: retail raw food and drinking water.

Table 2
Distribution of virulence genes among various *S. flexneri* serotypes from clinical and non-clinical (drinking water, retail raw milk, fruits, and vegetables) sources.

						0.95 Confidence intervals		
Variables	Total isolates, n(%)	Clinical isolates, n(%)	Non-clinical isolates, n(%)	Chi square	OR	Lower limit	Upper limit	p-value
ipaH								
2b	75(100)	53(70.6)	22(29.4)	3.64	0.519	0.263	1.023	0.05
1b	48(100)	36(75)	12(25)	0.30	0.806	0.376	1.726	0.5
2a	23(100)	16(69.6)	7(30.4)	1.04	0.608	0.233	1.588	0.3
7a	39(100)	38(97.4)	1(2.6)	10.7	13.96	1.859	104.87	0.001
1d	10(100)	9(90)	1(10)	0.89	2.65	0.326	21.51	0.34
Y	4(100)	3(90)	1(10)	0.019	0.848	0.086	8.367	0.888
ial								
2b	75(100)	53(70.6)	22(29.4)	0.536	0.75	0.357	1.6	0.463
1b	22(45.8)	14(29.2)	8(16.6)	1.224	0.58	0.222	1.52	0.26
2a	23(100)	16(69.6)	7(30.4)	0.189	0.805	0.302	2.14	0.66
7a	18(46.1)	18(46.1)	0	7.53	0	–	–	0.006
1d	0	0	0	0	0	–	–	0
Y	4(100)	3(90)	1(10)	0.006	1.09	0.1108	10.89	0.93
sat								
2b	75(37.7)	53(70.6)	22(29.4)	3.7	0.511	0.257	1.013	0.05
1b	48(24.13)	36(75)	12(25)	0.32	0.801	0.373	1.721	0.5
2a	23(11.4)	16(69.6)	7(30.4)	1.06	0.6	0.231	1.582	0.3
7a	39(19.6)	38(97.4)	1(2.6)	10.77	14	1.86	105.2	0.001
1d	10(5.0)	9(90)	1(10)	0.89	2.64	0.325	21.46	0.34
Y	0	0	0	0	0	–	–	0
set1A								
2b	67(89.3)	49(65)	18(24)	1.33	0.302	0.035	2.55	0.24
1b	1(2)	1(2)	0	0.33	0	–	–	0.5
2a	0	0	0	0	0	–	–	0
7a	0	0	0	0	0	–	–	0
1d	9(90)	8(80)	1(10)	1.008	2.88	0.336	24.66	0.31
Y	0	0	0	0	0	–	–	0
set1B								
2b	75(100)	53(70.6)	22(29.4)	2.287	0.523	0.22	1.219	0.13
1b	10(21)	8(16.6)	2(4.4)	0.114	1.31	0.265	6.554	0.73
2a	23(100)	16(69.6)	7(30.4)	0.545	0.68	0.254	1.861	0.46
7a	18(46)	18(46)	0	6.744	0	–	–	0.009
1d	1(10)	1(10)	0	0.325	0	–	–	0.56
Y	4(100)	3(90)	1(10)	0.0007	0.96	0.097	9.65	0.97

Note: p value < 0.05 was considered statistically significant. Clinical samples: Diarrheal stool, Non clinical samples: drinking water, retail raw milk, fruits, and vegetables.

S. flexneri, seven different virulence gene profiles were detected, designated as V1-V7 (Table 3). The most prevalent virulence patterns being that of V1 (34%) followed by V3 (29%), V4 (23.6%), V2 (6%), V5 (4.5%), V7 (2%), and V6 (0.5%). The current results also revealed that the virulence gene pattern showed a significant association among the strains of *S. flexneri* isolated from clinical and non-clinical samples (Table 3).

3.4. Virulence gene profiles among the different serotypes of S. flexneri

In addition, high heterogeneity was noticed in the virulence gene profiles among the different serotypes of *S. flexneri* isolated from human diarrheal stool and non-clinical samples. The current results also revealed that in serotype 2b the most predominant virulence gene pattern was V1(89.3%) followed by V3 (10.6%) while in serotype 1b the prevalent virulence gene pattern was V4 (54.2%) followed by V2 (25%) isolated from human diarrheal stool and non-clinical samples as shown in Table 4. Similarly in serotype 2a, 7a, 1d, and Y the prevalent virulence gene pattern was V3 (100%), V4 (53.8%), V5 (90%), and V7 (100%) respectively (Table 4). The only statistically significant association was observed between the serotype 1b and virulence pattern V2 isolated from clinical and non-clinical samples.

3.5. Association between virulence gene profiles and disease severity

The present investigation also revealed that the various virulence gene profiles are associated with disease severity (Table 5). About 32% ($p < 0.0001$) strains belongs to serotype 2b contain the complete set of

Table 3
Virulence profile analysis of *S. flexneri* isolates isolated from clinical and non-clinical (retail raw food and drinking water) sources.

									0.95 Confidence intervals		
Virulence pattern	*ipaH*	*ial*	*sat*	*set1A*	*set1B*	No. of isolates, n(%)	Chi square	OR	Lower limit	Upper limit	p-value
V1	+	+	+	+	+	68(34%)	74.97	4.21	2.98	5.94	<0.0001
V2	+	+	+	−	−	12(6%)	12.92	0.34	0.18	0.63	0.0003
V3	+	+	+	−	+	58(29%)	41.86	3.07	2.15	4.37	<0.0001
V4	+	−	+	−	−	47(23.6%)	16.51	2.11	1.46	3.06	0.00004
V5	+	−	+	+	−	9(4.5%)	18.072	0.25	0.12	0.49	0.00002
V6	+	−	+	−	+	1(0.5%)	36.02	0.025	0.003	0.18	<0.0001
V7	+	+	−	−	+	4(2%)	28.57	0.105	0.038	0.28	<0.0001

Note: p value < 0.05 was considered statistically significant.

Table 4
Distribution of predominant virulence gene patterns among the different serotypes isolated from different sources.

Serotypes (n)	Virulence Pattern, n(%)						
	V1	V2	V3	V4	V5	V6	V7
2b		–		–	–	–	–
Clinical (53)	49 (92.4)		4(7.5)				
Non-clinical (22)	18 (81.8)		4 (18.2)				
Total (75)	67 (89.3)		8 (10.6)				
1b					–	–	–
Clinical (36)	1(2.7)	6 (16.6)*	7 (19.4)	22 (61.1)			
Non-clinical (12)	0	6 (50)	2 (16.6)	4 (33.3)			
Total (48)	1(2)	12(25)	9 (18.7)	26 (54.2)			
2a	–	–		–	–	–	–
Clinical (16)			16 (100)				
Non-clinical (7)			7 (100)				
Total (23)			23 (100)				
7a	–	–			–	–	–
Clinical (38)			18 (47.4)	20 (52.6)			
Non-clinical (1)			0	1 (100)			
Total (39)			18 (46.2)	21 (53.8)			
1d	–	–	–	–			–
Clinical (9)					8 (88.8)	1 (11.2)	
Non-clinical (1)					1 (100)	0	
Total (10)					9(90)	1(10)	
Y	–	–	–	–	–	–	
Clinical (3)							3 (100
Non-clinical (1)							1 (100)
Total (4)							4 (100)

Note: (*) indicates P value < 0.05 and considered as statistically significant.

five virulence genes (*ipaH,ial,sat,set1A,set1B*), isolated from patients with high disease severity and 48.4% of the strains have different types of virulence patterns, in which fewer virulence genes were detected, where the related patients showed a moderate severity of the disease. Only 19.3% of the isolated strains showed mild severity of disease correlated with two different virulence profiles (Table 5).

4. Discussion

The present work is the first study addressing the distribution of *S. flexneri* virulence factors isolated from clinical and non-clinical (retail raw food and drinking water) samples and their association with serotypes in Pakistan. The study also highlights the statistically significant contribution of serotypes and virulence genes to the increased pathogenicity of shigellosis. Furthermore, a varied significant association was observed between various virulence genes isolated from clinical and non-clinical samples. In the current finding, the frequency and dissemination of five virulence genes were studied, and *ipaH* was highly prevalent in various serotypes of *S. flexneri* confirming various previous studies (Fan et al., 2017; Zhang et al., 2014). The presence of multiple copies of *ipaH*, chromosomal, and/or plasmid localized, might describe why the gene is present in all strains (Sangeetha et al., 2014).

The *ial* gene is also detected in most of the serotypes of *S. flexneri* and is located on the *inv* plasmid and is therefore vulnerable to spontaneous deletions (Bassa et al., 2010; Sangeetha et al., 2014). This is in contrast to *ipaH* which is located both on chromosomes and plasmids. The current data supports previous studies where the *ial* gene is detected in lower numbers compared to *ipaH* gene (Sangeetha et al., 2014; Thong et al., 2005).

The enterotoxin1 protein, encoded by *set1A* and *set1B* in *S. flexneri,* is located on the chromosome and was identified in 40% of all serotypes of *S. flexneri*. Previous data showed that the *set1* gene was merely found in 2a and 2b serotypes of *S. flexneri* (Niyogi et al., 2004; Sangeetha et al., 2014; Vargas et al., 1999) however in this study, many serotypes of *S. flexneri* tested positive to *set1* gene. This finding is consistent with the earlier reported study by Fan et al., 2017 in China (Fan et al., 2017). Both *set1A* and *set1B* were observed in 34% of *S. flexneri* isolates while only 4.5% of *S. flexneri* isolates had only one subunit (*set1A*) and 31.5% of *S. flexneri* isolates had *set1B*. So this study may probably explain that even a single subunit is enough for pathogenicity of *set1* gene. Another virulence-related gene is *sat* which was found in almost all serotypes of *S. flexneri* except serotype Y. A similar finding was also reported from Iran and Eastern China (Fan et al., 2017; Nave et al., 2016). Statistically, a significant difference was observed between the distribution of various virulence genes in some serotypes of *S. flexneri* isolated from clinical and non-clinical samples. The current study revealed that a high level of virulence gene was detected in serotype 2b isolated from both clinical and non-clinical samples suggests that serotype 2b is an emerging virulent shigellosis agent in the region.

Several virulence patterns were observed in this study. The most

Table 5
Association between virulence pattern and disease severity.

	Virulence pattern								0.95 Confidence intervals		
Disease severity	*ipaH*	*ial*	*sat*	*set1A*	*set1B*	Total isolates, n(%)	Chi square	OR	Lower limit	Upper limit	p-value
+++	+	+	+	+	+	50(32)	63.22	4.44	3.002	6.57	<0.0001
++	+	+	+	−	−	6(3.8)	12.06	0.25	0.1	0.58	0.0005
++	+	+	+	−	+	45(29)	44.26	3.62	2.43	5.4	<0.0001
++	+	−	+	−	−	15(9.6)	1.29	0.72	0.41	1.26	0.25
++	+	−	+	+	−	8(5)	8.72	0.34	0.16	0.72	0.003
++	+	−	+	−	+	1(0.6)	22.76	0.03	0.005	0.28	<0.0001
+	+	−	+	−	−	27(17.4)	3.91	1.57	1.001	2.48	0.04
+	+	+	−	−	+	3(1.9)	18.07	0.12	0.03	0.38	<0.0001

Note: p value < 0.05 was considered statistically significant. Disease severity score: Severe +++ (Abdominal pain, fever $\geq$39 °C, frequency of bloody stools $\geq$6 per day, frequency of vomiting 2–4 episodes per day), Moderate ++ (Abdominal pain, fever 38.5–38.9 °C, frequency of bloody stools 4–5 per day, frequency of vomiting episodes 1 per day), Mild + (Abdominal pain, fever 37.1–38.4 °C, frequency of watery stools 1–3 per day).

prevalent virulence pattern was V1, harboring the entire set of five virulence genes. This suggests that the presence of more virulence genes may explain the high severity of the disease. The current study also demonstrated the distribution of predominant virulence gene patterns among the different serotypes isolated from different sources. The predominant virulence pattern V1 that also contained the entire set of five virulence genes in serotype 2b of *S. flexneri,* isolated from clinical and non-clinical samples suggests a virulent aspect of non-clinical and clinical isolates among which 2b serotypes were shown to be more virulent. The present study revealed that most of virulence profile among various *S. flexneri* strains isolated from clinical and non-clinical sources showed same frequency this might indicate the genetic relatedness of virulence profile between clinical and non-clinical strains of *S. flexneri* serotypes.

This study demonstrated that various virulence patterns are associated with the severity of the disease. The presence of higher number of virulence genes proved to be an indication of increased clinical severity (Medeiros et al., 2017). This association with disease severity, including fever ≥39 °C, frequency of bloody stools ≥6 per day and 2–4 vomiting episodes per day showed an association with the V1 virulence gene profile. Interestingly we observed that 20 isolates of serotype 7a had the same virulence profile (*ipaH, sat*) but different clinical severity. Among these twenty isolates of serotype 7a, fifteen isolates showed increased clinical severity as compared to the other 5 isolates that showed decrease clinical severity. This may be due to the presence of mutation either in the *ipaH* or *sat* gene that might be important for detecting high or low virulence isolates.

In conclusion, this investigation revealed that serotype 2b was the most virulent strains isolated from both clinical and non-clinical samples. It also highlights the dissemination of virulence genes of *S. flexneri* serotypes isolated from clinical samples in Pakistan. It was observed that the concurrency of the various virulence gene profiles varied significantly, leading to diverse severities of the illness. The virulence genes profile associated with a serotype 2b in which high severity of disease was observed. This finding also suggests the existence of virulence genes in *S. flexneri* in clinical and non-clinical sources, which might be an important marker to improve epidemiological monitoring.

Author contributions

MQ, IN, AD, and JN study conceived and designed. IN wrote the main manuscript, performed the experiments. MQ, IN, AD, and JN analyzed the data. MQ, AD, FA, JN, MNS, and HR, review and commented on the manuscript. All authors read and approved the final manuscript.

Ethical approval

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. In addition, the institutional research ethical committee approved the present study.

Informed consent

Informed consent was obtained from all patients/guardian for being included in the study.

Declaration of Competing Interest

All the authors declare that they have no conflict of interest.

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