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Isolation of *Salmonella sp* that causes pathogenicity on fresh fruit juice sold along the roadside in Namakkal district

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Abstract
The nutritional value, mineral level, and vitamin content of fruit juices are widely known aspects of these beverages. In a great number of tropical nations, these beverages are considered to be the beverages of the common man and are sold in all public areas and roadside shops. Infections that are transmitted through food, such as salmonellosis, have become more prevalent in humans during the past few decades. A significant high prevalence of *Salmonella* was found in apple samples (80%), followed by Sapotta and watermelon (60%) and orange, Sathukudi, pomegranate, and molampallam (40%) isolates. The current investigation reveals the significant high prevalence of *Salmonella* in apple samples. The most powerful biofilm makers were *S. typhimurium*, followed by *S. typhi*, which produced forty percent of the total. *S. typhi* demonstrated the highest resistance to serum, surpassing *S. typhimurium* in terms of resistance. With the highest number of ESBL resistance genes, the three species of *Salmonella paratyphi A* were found to be followed by *Salmonella typhimurium*. All of the fresh fruit juices that were sold on the street in many different locations of the city were found to be contaminated with *Salmonella* species, according to the findings of the findings of the study. People should steer clear of fruit juices sold on the street. It is possible that the contamination of fruit juices could be reduced through the application of conventional hygienic practices and the education of the vendors regarding health.

Keywords: Fruit juices, biofilm, beta lactamase, ESBL gene, *Salmonella typhi*, *Salmonella paratyphi A* and *Salmonella typhimurium*

Introduction

There is little doubt that fruit juice is the most popular beverage in the world, particularly in India and the United States. The demand for fresh vegetables and fruit juices is consistently one of the highest in the industry. Due to the fact that the region is tropical, the average temperature remains high throughout the majority of the year (February through September), which results in an increased demand for certain goods. In accordance with Lewis *et al*., (2006) [14] and Indhu *et al*., (2017) [11], juices that are extracted by squeezing a variety of fruit juices, such as orange, grape, pomegranate, apple, pineapple, mango, and sapotta, among others, are served after being significantly diluted with ice and water.

The nutritional value, mineral level, and vitamin content of fruit juices are widely known aspects of these beverages. In a great number of tropical nations, these beverages are considered to be the beverages of the common man and are sold in all public areas and roadside shops. Having said that, due to the ease with which they can be consumed, as well as the speed with which they can be cleaned, handled, and extracted, they frequently pose a risk to the public's health. According to Parish (1997) [18] and Sandeep *et al*., (2001) [20], there have been instances of food-borne illnesses that have been linked to the intake of fruit juices in a number of locations across India and elsewhere.

It is possible for pathogenic organisms to infiltrate fruits and vegetables through damaged surfaces, such as punctures, wounds, cuts, and splits that occur during the growth process or during harvesting. Contamination from raw materials and equipments, additional processing conditions, improper handling, and the prevalence of unhygienic conditions are all factors that contribute significantly to the introduction of bacterial pathogens into juices that are prepared from these fruits or vegetables (Victorian Government Department of Human Services 2005; Oliveira *et al*., 2006; Nicolas *et al*., 2007; Indhu *et al*., 2014) [24, 17, 15, 10].

Numerous research conducted in a variety of nations have demonstrated that the microbiological quality of fruit juices is under question. Fruit juices were found to contain *Salmonella* species, *Klebsiella pneumonia*, and *E. coli*, according to Sandeep *et al*., (2001) findings. The consumption of contaminated fresh vegetables, fruits, and sprouts has been connected to a number of outbreaks of human gastroenteritis (Brackett and Splittstoesser, 1992; Poorna Viswanathan and Randhir Kaur, 2001; Sasikala *et al*., 2018) [3, 19, 21]. These outbreaks have been linked to the consumption of contaminated items.

In recent years, there has been a considerable increase in the development of ESBL in Enterobacteriaceae, particularly *Salmonella*, in a number of countries, including India. Integrons, which play a particularly important role in the development of multidrug resistance, can also include a major amount of antimicrobial resistance genes that are present on plasmids and transposons in Enterobacteriaceae (Wang *et al*., 2008) [25]. Integrons are thought to be responsible for the transmission of multidrug resistance. It has been demonstrated that the presence of such integrons can be predicted by resistance to sulfamethoxazole,

cotrimoxazole, gentamicin, tobramycin, ampicillin, piperacillin, and cefuroxime. According to research conducted by Dallenne *et al*., (2010) [6] and Thangaraj and Sivanantham (2015) [22], the ESBLs that are most frequent in Enterobacteriaceae are of the TEM-type, SHV-type, and CTX-M-type varieties.

Materials and methods

Collection of samples

At the stall of the street vendor selling fruit juices, freshly squeezed fruit juices were collected.

Isolation of *Salmonella*

One gram of each hard sample was homogenized in 25 milliliters of buffered peptone water (BPW) under aseptic circumstances for two minutes using a sterile homogenizer. At the same time, 25 milliliters of milk sample and fresh fruit juices were completely combined with two hundred and 25 milliliters as well.

Preparation of Slant

Following the preparation of the nutrient agar slants, the individual colonies that were picked from the streaked plates were then streaked onto the nutrient agar slant test tubes in a continuous manner throughout the experiment. Afterwards, the test tubes were placed in the refrigerator for additional research after being incubated at 37º C for sixteen to 24 hours.

Identification of *Salmonella spp*

Preliminary Tests: Gram staining, Motility test, Catalase test, Oxidase test.

Biochemical Tests: Indole test, Methyl red test, Voges – proskauer test

Determination of biofilm production

A solid medium brain heart infusion broth (BHI) that has been created specifically for this purpose and is supplemented with 5% sucrose and Congo red. All of the components that made up the medium were BHI, sucrose, agar, and congo red stain. After preparing a concentrated aqueous solution of Congo red, it was autoclaved at 121ºC for 15 minutes, while remaining separate from the other components of the medium. After the agar had cooled to 55º C, the Congo red was added.

Assay for beta lactamase production

Following the spot inoculation of the test organism's broth culture onto Mueller-Hinton agar and 1% starch, the mixture was then incubated at 370º C for an entire night. The plates were then flooded with phosphate buffered saline that had been freshly prepared and contained penicillin, potassium iodide, and iodine. The existence of clear, colorless zones surrounding the bacterial growth is a sign that beta lactamase is being produced, according to Lateef (2004) [13].

Cell surface hydrobocity

In a 250 ml Erlenmeyer flask, all of the isolates, including the standard strain, were cultured in nutritional broth (50 ml) while being shaken at a speed of 200 revolutions per minute. After being collected using centrifugation at a force of 10,000 × g for a duration of 15 minutes, the cells were washed twice in sterile phosphate-buffered saline with a pH of 7.1. Subsequently, the cells were suspended in the same buffer until they reached an initial optical density (OD) of about 1.0 (A0) at a wavelength of 600 nm. Subsequently, 300 ml of xylene was introduced into 3 ml of microbial suspension, and the mixture was vortexed for a duration of two minutes. At 600 nm, the optical density (OD) of the aqueous phase was measured after ten minutes had passed.

Serum inactivation assay

Glucose phosphate broth that contained bromothymol blue and two percent of human serum was used to inoculate the isolates, and the mixture was then heated to 370º C for 24 hours. Because of this, the color changed from green to yellow, which was the consequence that was witnessed.

Isolation of plasmid DNA

1.5 ml of a bacterial culture that has been cultivated for 24 hours contains plasmid. Following centrifugation of the cells in a micro centrifuge, the supernatant should be discarded. Add 100 ml of Solution A to the pellet. Include 100 ml of solution B. Add 100 µl of Solution C to the viscous substance that was previously mentioned. Invert the contents 4 or 5 times to ensure that they are well combined. The majority of the genomic DNA and other cell debris will precipitate into a thick clump. Centrifuge at a speed of 12000 rpm after 30 minutes. To precipitate DNA, simply add 150 µl of solution D and stir thoroughly.

Rotate the content at a speed of 12000 rpm for a period of 30 minutes. To remove the precipitate, discard the supernatant and dissolve it in 150µl of pure alcohol. Then, spin the mixture at a speed of 10000 rpm for a duration of 20%. Throw away the supernatant, and save the DNA pellets in a safe place. When necessary, dissolve the DNA in a dilution solution consisting of 20 ml (TE buffer). A confirmation of the isolated plasmid DNA was achieved by the use of agarose gel electrophoresis.

Polymerase chain reaction for ESBL gene amplification

The primers was obtained from Sigma, India and used in the PCR comprised Primer SHV F 5’ CTT TAT CGG CCC TCA CTC AA, and SHV R 5’ AGG TGC TCA TCA TGG GAA AG, TEM F 5’ CGC CGC ATA CAC TAT TCT CAG AAT GA’3 and TEM R 5’ ACG CTC ACC GGC TCC AGA TTT AT, CTXM F 5’ ATG TGC AGY ACC AGT AAR GTK ATG GC and CTXM R 5’ TGG GTR AAR TAR GTS ACC AGA AYC AGC GG ‘3. A sterile laminar flow hood is used to create the PCR mix, which is then placed in a thin-walled PCR tube. Each PCR reaction mixture, which consisted of 20 µl, was composed of 2 µl of template DNA (plasmid DNA), 2 µl of 10 X PCR buffer, 0.5 µl of each primer (0.5 µM), 1 µl of 0.2 mM of each deoxynucleotide triphosphate (dNTP'S), and 1 µl of Taq DNA polymerase (Con. 5U/ µl). Additionally, 8.0 µl of molecular grade water was included in the mixture.

Following the first denaturation at 95ºC for 15 minutes, the samples were put through 30 cycles of denaturation at 94ºC for 30 seconds, annealing at 62ºC for 1.30 and extension at 72ºC for 1 minute. The final extension was carried out at a temperature of 72ºC for 10 minutes. The reaction mixtures were subjected to electrophoresis on a 1.5% Agarose gel, which contained Ethidium bromide at a concentration of 0.2 mg/ml. This analysis was performed in the presence of a suitable DNA molecular weight marker. The aliquots, which were 20 µl in volume, were evaluated. After that, use a UV transilluminator to view the bands of amplification, and

check for the presence of a resistance gene by employing a marker.

Results

Isolation of Salmonella spp

An isolate of Salmonella was found in 19 out of 35 samples of fruit juice, which accounts for 54.2% of the total (Table 1). There were several samples that produced more than one isolate. For the purpose of obtaining the isolates, selective media and routine biochemical assays were utilized. Each and every isolate was validated through the use of selective media (SS agar) with simultaneous inoculation into the biochemical test. Salmonella was responsible for producing colonies that were large, circular, low convex, smooth, and colorless with a black center. The observed colony morphology of the isolates featured these characteristics. The results of the tests for MR, catalase, and citrate were positive for *Salmonella*, while the results for the tests for indole and urease were negative. Table 2 contains a tabular representation of the colony characteristics and biochemical data.

The current investigation reveals the significantly high prevalence of *Salmonella* in apple samples (80%), followed by Sapotta and watermelon (60%) and orange, Sathukudi, pomegranate, and molampallam (40%) isolates. The highest prevalence of *Salmonella* was found in watermelon. In this particular investigation, three distinct species of *Salmonella* were found to be present. These included *Salmonella typhi*, *Salmonella paratyphi A*, and *Salmonella typhimurium*. Within this group, the *Salmonella parapyphi A* strain had the highest incidence (34.2%), followed by the *Salmonella typhimurium* strain (25.7%), and then the *Salmonella typhi* strain (14.2%). Both *Salmonella paratyphi A* and *Salmonella typhimurium* were found in large concentrations in Apple and Sapotta, with the former contributing sixty percent. According to the findings of this experiment, every variety of fruit juice contained at least one isolated strain of *salmonella*. There were seven different kinds of fruit juice, and three of them, apple, sapotta, and sathukudi, were found to be contaminated with *Salmonella* species.

Biofilm formation

The total percentage of biofilm producers among the isolates was 65.3%, with 38.4% of the isolates being considered to be strong biofilm producers. The most powerful biofilm makers were *S. typhimurium*, followed by *S. typhi*, which produced forty percent of the total. Among the seven different kinds of fruit juices, the pomegranate juice had the highest biofilm producers, followed by the orange juice, and then the watermelon juice (Table 3).

Betalactamase production

Betalactamase was produced by 73% of the isolates, with 30% of *S. typhimurium* exhibiting betalactamase activity. *S. paratyphi* and *S. typhi* showed the second highest levels of betalactamase activity, with 26.6% and 20% respectively. In the case of fruit juice, the largest betalactamase producers were found in orange and pomegranate juice (about 40%), followed by sapotta juice (about 36.6%) (Table 3).

Serum resistance

A total of 53.8% of the isolates in this investigation exhibited serum resistance. Among them, *S. typhi* exhibited the highest level of resistance to serum, followed by *S. typhimurium's* second highest level. Orange was the source of the most serum-resistant isolates, followed by sathukudi as the second most resistant. (Table 3)

Amplification of ESBL genes

Amplification of ESBL genes using multiplex PCR was the final step in our work, which was conducted in the present setting. For the purpose of amplifying antibiotic resistance, four ESBL primers were included in the experiment. 69 point 2% of the isolates included at least one ESBL gene. The highest number of ESBL resistance genes was found in *Salmonella paratyphi A*, which was followed by *Salmonella typhimurium* among the three species involved. SHV had the highest prevalence of ESBL genes in our isolates, which was 40%, according to this study. TEM with the second highest percentage (27.7%), followed by OXA and TEM with a 25% share. Table.4

Discussion

Contamination of food with bacteria that are resistant to antibiotics poses a significant risk to public health. This is because the determinants of antibiotic resistance can be passed on to other pathogenic bacteria, which might possibly compromise the treatment of serious bacterial diseases. Over the course of the last few decades, there has been a rise in the incidence of antibiotic resistance among food-borne diseases (Angulo *et al*., 2000; Chiu *et al*., 2002; Devies *et al*., 1999; Garau *et al*., 1999). [1, 4, 7, 9]

Millions of people in India enjoy fruit juices, meals, and snacks that are supplied by street food sellers. These items are consumed rather frequently. These street foods offer a supply of nutrients that is accessible to a large portion of the population at a cheap price. According to the Food and Agriculture Organization of the United Nations (1988) and Ohiokpehai (2003) [16], people favor fruit drinks sold on the street because of their flavor, low cost, and availability at the appropriate time. According to Barro *et al*., (2006) [2], the inappropriate handling and serving procedures of street foods are frequently connected with the development of diarrheal disorders caused by the foods themselves.

Therefore, the conditions under which street food is prepared and sold on the street cause a great deal of concern for the health of the consumers. The majority of the time, vending locations do not have access to running water; therefore, washing one's hands and utensils is typically done in one or more buckets, and occasionally without the use of soap. The disposal of wastewaters and debris in the vicinity provides insects and rodents with the nutrition they need to survive. There are some juices that do not have adequate protection against flies, which are known to carry infections that are transmitted through food. It is the purpose of this study to determine the hygienic quality of juices sold on the street and to determine the impact that these juices have on the contamination of street foods.

According to the findings of our research, the quantity of *Salmonella* that is found in fresh fruit juices is extremely high. A total of 35 distinct fruit juices were gathered from the area surrounding the Namakkal district. 54.2% of the *Salmonella* species were found in the 35 samples that were taken. Within this group, the *Salmonella parapyphi A* strain had the highest incidence (34.2%), followed by the *Salmonella typhimurium* strain (25.7%), and then the *Salmonella typhi* strain (14.2%). Apple samples had the highest prevalence of *Salmonella*, which was 80%, followed

by sapota and watermelon, which had 60%. According to the findings of this experiment, every variety of fruit juice contained at least one isolated strain of salmonella. There were seven different kinds of fruit juice, and three of them, apple, sapotta, and sathukudi, were found to be contaminated with *Salmonella* species.

The biofilm-causing isolates were extremely challenging to treat due to their high level of resistance to antibiotic treatment. It poses a significant challenge to those who work in the medical field and poses a serious threat on a global scale (Jegadeeshkumar *et al*., 2010) [12]. Isolates were found to display a number of virulence factors and an improved resistance against phagocytosis and other host defensive mechanisms throughout the process of biofilm development (Costerton *et al*., 1999)[5]. During the course of this research, a total of 65.3% of the isolates produced biofilm, with 38.4% of those isolates being considered to be significant biofilm producers. *S. typhimurium* was the most powerful generator of biofilm, followed by *S. typhi*, which produced forty percent of the total. Pomegranate juice had the highest biofilm producers out of the 7 different types of fruit juices, followed by orange juice, and then watermelon juice.

Amplification of ESBL genes from fruit juice isolates of *Salmonella* spp. was the final step in this experiment because it was the last part of the investigation. With the exception of TEM-1, TEM-2, and SHV-1, the majority of ESBLs have developed through the process of genetic mutation from native ß-lactamases. Some gram-negative bacteria, particularly those belonging to the Enterobacteriaceae family, are known to contain these parent enzymes. 69.2% of the isolates included at least one ESBL gene. The highest number of ESBL resistance genes was found in *Salmonella paratyphi A*, which was followed by *Salmonella typhimurium* among the 3 species involved. According to the findings of this study, the ESBL-positive *salmonella* species studied primarily encoded SHV (40%) and TEM (27%).

It is our understanding that this is the first time that an ESBL has been reported in *Salmonella* in the Namakkal District. There are no research that have been reported on ESBL genes derived from *Salmonella* species isolated from fruit juice. The majority of the people who worked in the kitchens to manufacture fruit juice were extremely young folks, including children, according to this study. In accordance with the findings of a previous study conducted in Namakkal town (Valli *et al*., 2010)[23], the majority of the individuals possessed low levels of education and lacked experience.

Conclusion

At the end of the day, the findings of the study reveal that every single fresh fruit juice that was sold on the street in a number of different areas of the city was contaminated with *Salmonella* species. It is being argued that the primary cause of contamination is the low quality of the water that is being used for dilution, in addition to the prevalent unsanitary conditions that are associated with the washing of utensils and the maintenance of the premises. Whether it be by the side of a busy road with heavy automobile traffic (airborne particles) or by the side of the waste disposal system and overcrowding, the site appears to contribute to the contamination. It is imperative that Government Health Agencies take the necessary steps to educate sellers on properly hygienic and safe food procedures, as well as to establish and enforce appropriate norms for street food vending. Avoiding fruit juices sold on the street is recommended. By educating the sellers about health and implementing acceptable hygienic practices, it may be possible to limit the amount of contamination that occurs in fruit juices.

Table 1 Prevalence of *Salmonella* spp from fruit juices

S. No	Sample	Isolates
1	Apple	*S.typhimurium*
2	Orange	-
3	Sathukodi	*S.typhimurium,S.typhi*
4	Pomegranate	-
5	Sapota	*S.paratyphiA, S.typhimurium*
6	Watermelon	*S.paratyphiA*
7	Molampalam	-
8	Apple-2	*S.paratyphiA*
9	Orange-2	*S.paratyphiA, S.typhimurium*
10	Sathukodi-2	-
11	Pomegranate-2	*S.paratyphiA*
12	Sapota-2	*S.typhimurium S.paratyphiA*
13	Watermelon-2	-
14	Molampalam-2	*S.paratyphiA*
15	Apple-3	*S.paratyphiA, S.typhi*
16	Orange-3	-
17	Sathukodi-3	*S.paratyphiA*
18	Pomegranate-3	-
19	Sapota-3	-
20	Watermelon-3	*S.paratyphiA*
21	Molampalam-3	*S.typhimurium*
22	Apple-4	*S.typhi, S.paratyphiA*
23	Orange-4	-
24	Sathukodi-4	-
25	Pomegranate-4	-
26	Sapota-4	*S.typhimurium, S.typhi*
27	Watermelon-4	*S.typhimurium*
28	Molampalam-4	-
29	Apple-5	-
30	Orange-5	*S.typhimurium*
31	Sathukodi-5	*S.typhi*
32	Pomegranate-5	*S.paratyphiA*
33	Sapota-5	-
34	Watermelon-5	-
35	Molampalam-5	-

Table 2: Biochemical characters of *Salmonella* species

S. No	Test	***Salmonella* species**		
		S. typhi	***S.typhimurium***	***S.paratyphi A***
1.	Colour of the colony in SSA medium	Black	Colourless with black center	Black
2.	Gram staining	Gram negative	Gram negative	Gram negative
3.	Motility	Motile	Motile	Motile
4.	Indole	Negative	Negative	Negative
5.	Methyl Red	Positive	Positive	Positive
6.	Voges Proskauer	Negative	Positive	Negative

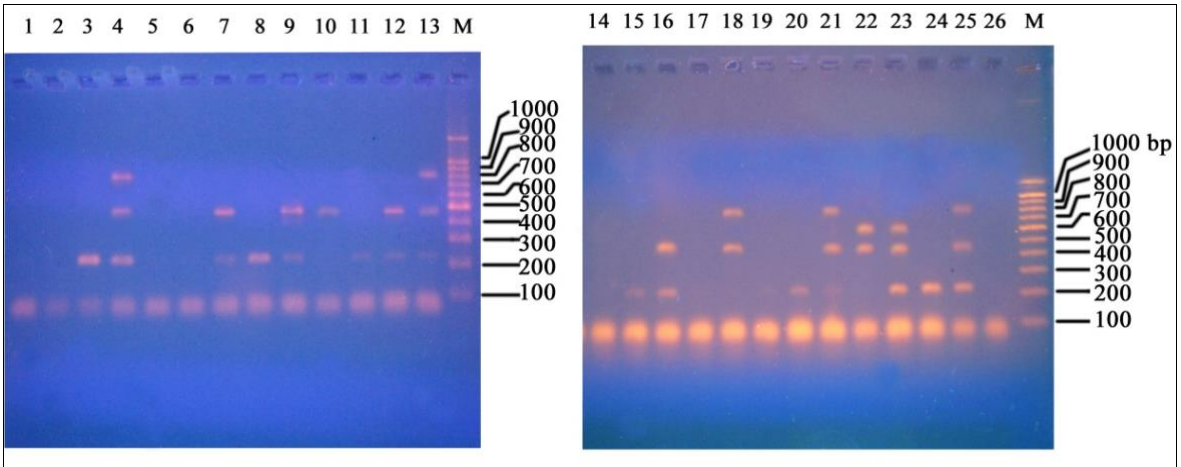
7.	Catalase	Positive	Positive	Positive
8.	Citrate	Negative	Positive	Negative
9.	Urease	Negative	Negative	Negative
10.	TSI	Acid butt/ alkaline slant no gas, speck of H2S.	Acid butt/ alkaline slant with gas, no H2S.	Acid butt/ alkaline slant with gas and no H2S.

Table 3: Virulence factors of *Salmonella spp*

S.no	Name of the samples	Biofilm (%)			Betalactamase (%)			Serum resistant (%)		
		Salmonella typhi	*Salmonella typhimurium*	*Salmonellapar atyphi A*	*Salmonella typhi*	*Salmonela typhimurium*	*Salmonella paratyphi A*	*Salmonella typhi*	*Salmonella typhimurium*	*Salmonella paratyphi A*
1	Apple	20	20	-	20	20	40	-	20	-
2	Orange	-	40	-	-	40	-	-	40	-
3	Sathukodi	20	20	20	-	20	20	40	20	20
4	Pomegranate	-	-	40	-	-	40	-	-	20
5	Sapota	20	40	20	20	60	20	20	20	20
6	Watermelon	-	20	40	-	20	20	-	20	-
7	Molampalam	-	20	20	-	20	20	-	-	20

Table 4

S. No	Name of the samples	TEM %			SHV %			OXA %			CTXM %		
		Salmonella typhi	*Salmonella typhimurium*	*Salmonella paratyphi A*	*Salmonella typhi*	*Salmonella typhimurium*	*Salmonella paratyphi A*	*Salmonella typhi*	*Salmonella typhimurium*	*Salmonella paratyphi A*	*Salmonella typhi*	*Salmonella typhimurium*	*Salmonella paratyphi A*
1	Apple	25	-	25	50		50	-	-	-	-	-	-
2	Orange	-	25	-	-	50	25	-	-	-	-	-	-
3	Sathukodi	25	-	-	50	-	25	25	-	-	-	-	-
4	Pomegranate	-	-	25	-	-	-	-	-	-	-	-	-
5	Sapota	25	25	50	-	50	50	-	25	25	25	-	-
6	Watermelon	-	25	-	-	25	-	-	-	-	-	25	-
7	Molampalam	-	25	25	-	-	25	-	25	25	-	-	-



1. ASty1, 2.SSty2, 3.SSt1, 4.SASp1, 5.SASty3, 6.WSp2, 7.ASp3, 8.OSp4, 9. OSty4, 10.PSp5

11.SASty5, 12.SASp6, 13.MSp7,14. ASp8, 15.ASt2, 16.SSp9, 17.WSp10,18.MSty6, 19.ASt3,

20.ASp11, 21.SASty7, 22.SASt4, 23.WSty8 24.OSty9,25.SSt5, 26.PSp12

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