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Public health risks associated with *Salmonella* contamination of imported edible betel leaves: Analysis of results from England, 2011–2017

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ABSTRACT

Fresh betel leaves (*Piper betle* L.), imported into the UK are a traditional ready-to-eat food consumed by Asian populations. We report here the consolidation of routinely collected data to model the public health risks from consumption of this food. Amongst 2110 samples collected at Border Inspection, wholesale, catering or retail, *Salmonella* was detected in 488 (23%) of samples tested between 2011 and 2017 and was the most commonly Salmonella-contaminated ready-to-eat food examined by Public Health England during this period. Using data from multiple samples (usually 5) tested per consignment sampled at Border Inspection, contamination levels were calculated by most probable number: seasonal, temporal and country specific differences were detected. Quantitative contamination data was used to estimate the levels present at retail, and a β-Poisson dose response model the probability of illness was calculated. Using data for products imported from India, the probability of acquiring infection following a single exposure (comprising of a single leaf) was estimated to be between 0.00003 (January–March) and 0.0001 (July–September). Using British Asian population data for individuals over 30 years of age in England in 2011, two estimates of consumption were modelled as 2.1 and 12.8 million servings per annum. Results from the model estimated 160 cases (range 102 to 242) and 960 cases (range 612 to 1456) per year in England for the two consumption estimates and equated to 34 (range 22 to 51) and 204 (range 130 to 310) salmonellosis cases per year reported to national surveillance. *Salmonella* from 475 of the contaminated samples were further characterised which showed a heterogeneous population structure with 46 *S. enterica* subsp. *Enterica* serovars, together with *S. enterica subs diarizonae* and *salamae* identified. Isolates from individual consignments were diverse and close genetic relationships between independent isolates were very rare except from within an individual consignment. There were no outbreaks detected as associated with betel leaf consumption. However analysis by whole genome sequencing of the 2014–17 data identified two cases where the clinical isolate had < 5 single nucleotide polymorphism differences to isolates from betel leaves which is indicative of a likely epidemiological link and common source of contamination. Due to the diversity of the *Salmonella* contaminating this product, associations between salmonellosis cases and betel leaf consumption will appear sporadic and unlikely to be detected by current surveillance strategies based on outbreak detection.

1. Introduction

Betel leaves (*Piper betle* L.), also known as paan leaves, are a traditional ready-to-eat food which South-eastern Asian populations combine with various fillings and chew after meals as a mild stimulant and breath-freshener. Consumption of betel quid (betel leaves with

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areca nut) has been associated with oral carcinoma, even in the absence of tobacco (Gupta and Johnson, 2014). Betel leaves also contain microbiological hazards and contamination with *Salmonella* has been reported previously in India and Bangladesh (Fakruddin et al., 2017; Haque et al., 2017; Nahar et al., 2018; Singh et al., 2006). Furthermore, consumption of this food has been linked to typhoid fever in India as well as in Cambodia (Singh et al., 2006; Singh et al., 2012).

Following a chance observation in England in 2011 of *Salmonella* contamination of betel leaves on retail sale, sampling and testing of betel leaves was incorporated into the UK's risk-based national monitoring programme of imported foods. Additional sampling was also carried out as part of increased levels of official controls as required under EU legislation (EU Regulation (EC) No 669/2009) at airport Border Inspection Post (BIP) Designated Points of Entry. The sampling regime for betel leaves at BIPs has been further enhanced in England by the testing of samples collected from retail sale including as part of national coordinated studies (McLauchlin et al., 2018; Willis et al., 2015).

Reporting of the isolation of *Salmonella* from human clinical specimens to Public Health England (PHE) as the designated public health authority is a statutory requirement in England (The Health Protection Act (Notification) Regulations 2010). The surveillance system also involves the voluntary submission of *Salmonella* isolates from clinical diagnostic samples to PHE for further characterisation: of the approximate 8500 salmonellosis cases reported each year in England, isolates are submitted for characterisation from about 95% of these cases (PHE unpublished data). Voluntary submission to the PHE Gastrointestinal Bacteria Reference Unit (GBRU) of *Salmonella* isolates from food and environmental samples tested in England also takes place.

In April 2014, whole genome sequencing (WGS) was implemented by PHE for the routine characterisation of isolates of *Salmonella* submitted to GBRU and since April 2015, this has been the primary typing tool used for public health surveillance. Multi Locus Sequence Typing (MLST) is used to assign each isolate a profile or sequence type, which has now replaced 95% of the serotyping previously undertaken since there is a 96% concordance for *Salmonella enterica* subspecies I serovars (Ashton et al., 2016). Hierarchical single linkage clustering is performed on pairwise single nucleotide polymorphism (SNP) distances, allowing quantification of the genetic relatedness between isolates and identification of microbiological clusters at pre-determined SNP thresholds of genetic relatedness. WGS therefore provides a high throughput, accurate, robust and reliable typing method which is well suited to routine public health surveillance (Ashton et al., 2016; Waldram et al., 2018a). Since *Salmonella* is a major foodborne pathogen in the UK, these tools are applied to both isolates derived from patients and those recovered from food to enable prospective detection and investigation of associations to elucidate the source of contamination.

The purpose of this report is to further report on the presence of *Salmonella* in imported fresh betel leaves sampled in England and to assess the risks posed by contamination of this product. This has been done by quantifying the occurrence of *Salmonella* recovered from imported betel leaves which were tested by PHE at border inspection as well as those at retail (including from coordinated studies we have previously reported; McLauchlin et al., 2018; Willis et al., 2015). This quantitative data was used in a dose response model to estimate the numbers of cases resulting from the consumption of this product. A combination of serotyping and sequence typing (ST) was used to assess the heterogeneity of the *Salmonella* contamination and this was further enhanced by comparison of the sequence data of *Salmonella* derived from foods with those recovered from patients with salmonellosis reported in England during the sampling period. This report illustrates the importance of the analysis and integration of multiple routinely collected data and the benefits of these types of analyses for communicable disease epidemiology, risk assessment, disease prevention and control as well as assisting in policy decisions on control of imported food.

2. Materials and methods

2.1. Sample collection and isolation of Salmonella

Samples of imported leaves (at least 50 g) were collected by Port Health Officers or sampling officers in either Environmental Health Departments or Port Health Authorities in accordance with the Food Standards Agency Food Law Practice Guidance (Food Standards Agency, 2015) between July 2011 and December 2017. No samples were included that were known to have been collected during investigations of outbreaks of foodborne illness.

Twenty five gram samples of betel leaves were examined for *Salmonella* in Public Health England Food Water and Environmental (FW&E) Laboratories located at Birmingham, London, Porton, Preston or York using the EN/ISO 6579 method (International Organisation for Standardisation, 2002). When testing samples collected at BIPs, individual consignments were defined by the codes used in the export certificates, and multiple samples (usually five 25 g samples) were tested from individual consignments. Each 25 g sample was independently tested for the presence of *Salmonella* and no pooling was used in this process. For betel leaf samples collected at retail, catering or wholesale, an individual 25 g sample was tested. Data for each individual sample were entered onto the FW&E Laboratory Information Management System and extracted onto Excel spreadsheets for analysis. Samples collected at retail, catering or wholesale as part of national coordinated studies were included in this analysis and comprised 22 samples in 2014 and 77 in 2017 (McLauchlin et al., 2018; Willis et al., 2015). The identification of *Salmonella* was performed at each individual FW&E laboratory and *Salmonella* cultures, where isolated, were sent to the GBRU for confirmation of identity and characterisation. Generally, only a single *Salmonella* culture from an individual sample was submitted to GBRU.

2.2. Submission and characterisation of Salmonella cultures

Salmonella isolates from food or clinical diagnostic specimens were sent to GBRU and confirmation of identity and serovar was performed by conventional serotyping for cultures submitted between 2011 and 2013 (Bale et al., 2007; Grimont and Weill, 2007). In 2014 both phenotypic and WGS methods were used and from 2015, WGS (Ashton et al., 2016) was the primary characterisation tool used: some cultures were tested by this method prior to 2014 as part of the evaluation of WGS implementation. DNA sequencing of *Salmonella* was performed by the PHE Genome Sequencing and Development Unit using Nextera library preparation and Illumina HiSeq 2500 (Illumina Inc., Albany, USA) in fast-run mode according to the manufacturer’s instructions. eBURST group (eBG), multilocus sequence type (ST), serovar and SNP profiles were derived from the genome data as described previously (Ashton et al., 2016).

A case of salmonellosis was defined as a patient for which a confirmed *Salmonella* was isolated from a clinical specimen in England and submitted to GBRU. Microbiological results and basic epidemiological data associated with all salmonellosis cases where a *Salmonella* was submitted to GBRU for characterisation were combined in PHE’s central enteric pathogen database, the Gastro Data Warehouse (GDW). This database also includes microbiological typing results from all food isolates submitted to GBRU. Data was extracted from the GDW onto Excel spreadsheets and combined with FW&E metadata associated with the food isolates. Sequence data from *Salmonella* isolated from both clinical cases and from betel leaves were compared according to isolates' eBG, ST and single linkage clustering at the 5-single nucleotide polymorphism (SNP) level (Dallman et al., 2018).

Where multiple cultures from the same sample or consignment collected at BIPs were characterised, one isolate per serovar per consignment was selected and this was designated as the representative isolate. Similarly, for samples collected at wholesale, catering or retail, where multiple isolates from the same sample were characterised, only one representative isolate per serovar per sample was included. The selection of representative isolates allowed assessment of distributions of *Salmonella* serovars from different countries of origin amongst the samples tested.

2.3. Quantification of Salmonella and estimated numbers of cases resulting from the consumption of betel leaves

For the contaminated consignments tested at BIPs where multiple samples were tested per consignment, *Salmonella* contamination levels (most probable number, MPN) were estimated using a generalised linear model with a binomial error and complementary log-log-link. This model was also used to compare the contamination levels according to year, quarter and country with effects reported as fold differences relative to the baseline of: samples collected during 2011 to 2012; samples collected between January and March; and samples with a country of origin from India. The model was then used to estimate the MPN with a 95% confidence interval by country and quarter and, assuming organisms are randomly distributed on leaves in each consignment, the probabilities of at least one organism being on a leaf were calculated using an average leaf weight of 6.08 g which was obtained by direct observations in the laboratory (mean of 13 leaves, range 5.18 to 6.82 g).

The MPN estimate for the probability of a leaf having at least one *Salmonella* was used to estimate a probability of infection using a β-Poisson dose response model as described previously (World Health Organization, Food and Agriculture Organization, 2002) using parameters for the expected level as well as the 2.5th and 97.5th percentiles. To capture uncertainty, the lower risk estimate (from the 2.5th percentile) was based on the lower end of the 95% confidence interval for the MPN and the upper estimate (from the 97.5th percentile) on the upper end of this interval.

To estimate the differences between contamination rates at border inspection and at retail the rate of contamination per sample was compared using a logistic regression model (with an identity link to estimate risk ratios). The outcome variable was occurrence of *Salmonella* and the effect of border versus retail was adjusted in the model for year, quarter and country of origin. An estimate of the numbers of servings consumed was made using population data (ONS DC2101EW ethnic groups Asian British Indian, Pakistani and Bangladeshi) and this was used to model the estimated numbers of cases based on the probability of infection. The numbers of cases reported to national surveillance was estimated using data from the infectious intestinal disease study (Tam et al., 2012) where a factor of 4.7 salmonellosis cases occurring in the community equated to one case reported to national surveillance.

3. Results

3.1. Salmonella detected and quantification in samples collected at Border Inspection Posts (BIPs)

A total of 1890 betel leaf samples were collected from 378 consignments sampled from BIPs located in four different airports in England during 2011 to 2017 (Table 1). Between 1 and 28 replicate 25 g samples were independently tested from each consignment with five samples being tested for 335 (89%) of the consignments. The number of samples tested each year varied between 16 and 539 samples (6 to 108 consignments), where the highest number of both samples and consignments were tested between July and December 2011. *Salmonella* were recovered from 455 (24%) of the samples and in 214 (57%) of the consignments at the point of import (Table 1). *Salmonella* were detected in samples collected during all years.

Table 1
Salmonella contamination detected in betel leaves collected at Border Inspection Posts in four English airports and at retail, wholesale or catering: from 2011 to 2017.

Year	Collected at Border Inspection Posts				Samples collected at retail, wholesale or catering[1]	
	Number of samples		Number of consignments			
	Total	*Salmonella* detected (%)	Total	*Salmonella* detected (%)	Total	*Salmonella* detected (%)
2011	539	198 (37%)	108	83 (77%)	67	17 (25%)
2012	515	120 (23%)	103	62 (60%)	0	
2013	481	78 (16%)	97	42 (43%)	11	1 (9%)
2014	145	16 (11%)	24	8 (33%)	42	1 (2%)
2015	116	24 (21%)	19	9 (47%)	22	3 (14%)
2016	78	19 (24%)	21	10 (77%)	1	0
2017	16	0	6	0	77	11 (14%)
Total	1890	455 (24%)	378	214 (57%)	220	33 (15%)

[1] 188 samples from retail, 31 from wholesale and 1 from catering

Table 2
Countries of origin and *Salmonella* contamination rates in betel leaves collected at Border Inspection Posts in four English airports, and at retail, wholesale or catering: 2011–2017.

Country of origin	Collected at Border Inspection Posts				Collected at retail, wholesale or catering	
	Number of samples		Number of consignments		Number of samples	
	Total	*Salmonella* detected (%)	Total	*Salmonella* detected (%)	Total	*Salmonella* detected (%)
Bangladesh	1064	278 (26%)	207	133 (64%)	78	22 (28%)
India	415	70 (17%)	82	38 (46%)	66	5 (8%)
Malaysia	65	17 (26%)	17	8 (47%)	2	0
Nepal	5	1 (20%)	1	1 (100%)	0	
Sri Lanka	23	2 (9%)	6	2 (33%)	11	2 (18%)
Thailand	135	23 (17%)	30	10 (38%)	2	0
Other	0		0		3[ø]	0
Not known	183	64 (35%)	35	60 (63%)	58	4 (7%)

[ø] Other = Indonesia, Pakistan and Qatar.

Information allowing identification of the country of origin was available for 1707 (90%) of the samples and 343 (91%) of the consignments, and these were identified as imported into the UK from six different countries: 85% (1614) of the samples came from either Bangladesh, India or Thailand (Table 2). *Salmonella* were isolated from betel leaves originating from all countries identified, and contamination rates per sample varied between 9 and 26% (Table 2). The 70 *Salmonella* contaminated samples from India sampled at BIPs pre-dated the 2016 EU wide statutory control requirements.

Based on the date of collection, there was a lower contamination rate during the first three months of the year compared to remainder of the year for both samples and consignments collected at the BIPs (Table 3).

The MPN model for the consignments collected at the BIPs was used to estimate the levels of contamination (Fig. 1) and using a fold difference to the 2011 to 2012 level as a baseline, there were no significant differences between the levels for samples collected in 2011–14, and 2015–17 (Table 4). This model did however show significantly lower levels of contamination in the first three months of the year as compared to the other three quarters (using January to March as a baseline). In addition, using all results from India as a baseline, there were significantly higher levels of contamination in samples collected from Bangladesh during 2011 to 2012 as well as from Malaysia and where the country of origin was not known (Table 4).

Using an average weight of an individual leaf as 6.08 g, MPNs were used to calculate the probability of a single leaf having at least one *Salmonella* (Figure 1). Based on random mixing the chance of 2 or more

Table 3
Salmonella contamination rates and period of sampling of betel leaves collected at Border Inspection Posts in four English airports and collected at retail, wholesale or catering: 2011–2017.

	Period of sampling (based on the date of collection)			
	January–March	April–June	July–September	October–December
Collected at Border Inspection Posts				
Total numbers of samples	500	278	526	586
Salmonella detected (%)	49 (10%)	62 (23%)	155 (29%)	188 (32%)
Total numbers of consignments	96	57	105	120
Salmonella detected (%)	37 (39%)	28 (49%)	71 (68%)	78 (65%)
Collected at retail, wholesale or catering				
Total numbers of samples	41	42	93	42
Salmonella detected (%)	2 (5%)	8 (19%)	23 (25%)	1 (2%)

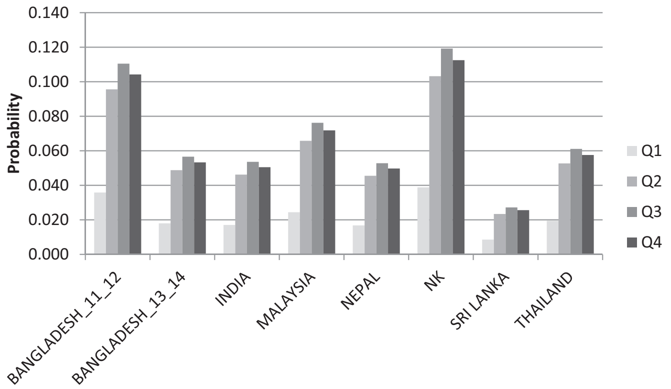

Fig. 1. Estimated probability of a betel leaf having at least one *Salmonella* by quarter and country of origin using a most probable number technique.
Q1 = January to March; Q2 = April to June; Q3 = July to September; Q4 = October to December.

Salmonella on an individual leaf was very low in any country or season ($p < 0.007$).

3.2. *Salmonella* detected in samples collected at wholesale, catering or retail

A total of 220 betel leaf samples were collected at either retail (188 samples), wholesale (31 samples) or from catering (one sample): *Salmonella* were recovered from 33 (15%) of these samples (Table 1). The numbers of samples collected each year varied between zero in 2012 and 77 in 2017 where a co-ordinated national survey was performed (McLauchlin et al., 2018). Contamination with *Salmonella* was detected in all years except from 2012 (when no samples were collected) and 2016 (where only one sample was collected; Table 1).

Amongst all 220 samples collected from wholesale, catering or retail, the country of origin was identified for 162 (74%): eight different countries were identified of which 155 samples (68%) came from Bangladesh, India or Sri Lanka. The contamination rate for these three countries varied between 8 and 28% (Table 2).

Based on the date of sample collection, for all samples collected at wholesale, catering and retail there was a lower contamination rate during the first three months of the year and this was similar to the samples collected at the BIPs (Table 3). Results from the logistic regression model gave an estimate of the presence of *Salmonella* being 32% lower at retail than at the point of border inspection and this was significant (95% confidence interval 3% to 52% lower, $p = 0.03$). The contamination levels for products at retail was used to represent exposure during consumption, and these quarterly levels were used in the subsequent section to model the probability of infection.

3.3. Application of dose response relationship and modelling the numbers of salmonellosis cases

Based on consumption of a single leaf and using the estimated dose response model, the probability of salmonellosis by quarter and country was estimated (Fig. 2). The risk of becoming ill was highest in quarter 3 and lowest in quarter 1 for all countries (Fig. 2) and the comparative risk varied between a factor of 3 (samples from Bangladesh in 2013–14) to 3.5 (samples from India, Nepal and Sri Lanka).

Contamination of leaves from India was used as a representative set of results for subsequent analyses since this was based on the second highest number (22%) of samples and was not significantly different from other countries apart from Bangladesh during 2011 to 2012, Malaysia and where the country of origin was not known (Table 4). The

Table 4
Fold differences in most probable number estimations by year, quarter and country of origin.

Factor	Category	Samples	*Salmonella* detected (%)		MPN per 25 g sample (95% CI)	Fold effect (95%CI)	aFold effect (95%CI)*
Year	2011–2012	1054	318	(30%)	0.359 (0.322–0.401)	Baseline	Baseline
	2013–2014	626	94	(15%)	0.163 (0.133–0.199)	0.45 (0.36–0.57)	1.08 (0.72–1.61)
	2015–2017	210	43	(20%)	0.229 (0.170–0.309)	0.64 (0.46–0.88)	0.66 (0.41–1.06)
Quarter	January to March	500	49	(9.8%)	0.103 (0.078–0.136)	Baseline	Baseline
	April to June	278	63	(22.7%)	0.257 (0.201–0.329)	2.49 (1.71–3.62)	2.74 (1.87–4.02)
	July to September	526	155	(29.5%)	0.349 (0.298–0.409)	3.38 (2.45–4.67)	3.44 (2.45–4.81)
	October to December	586	188	(32.1%)	0.387 (0.335–0.447)	3.75 (2.74–5.14)	3.05 (2.19–4.26)
Country	Bangladesh 2011–2012	744	237	(31.9%)	0.384 (0.337–0.436)	2.08 (1.59–2.71)	2.00 (1.44–2.79)
	Bangladesh 2013–2014	320	41	(12.8%)	0.137 (0.101–0.186)	0.74 (0.5–1.09)	0.93 (0.59–1.46)
	India	415	70	(16.9%)	0.185 (0.146–0.234)	Baseline	Baseline
	Malaysia	65	17	(26.2%)	0.303 (0.188–0.489)	1.64 (0.96–2.79)	2.07 (1.07–3.98)
	Nepal	5	1	(20.0%)	0.223 (0.031–1.591)	1.21 (0.17–8.73)	1.35 (0.18–10.04)
	Sri Lanka	23	2	(8.7%)	0.091 (0.023–0.364)	0.49 (0.12–2.01)	0.47 (0.12–1.93)
	Thailand	135	23	(17.0%)	0.187 (0.124–0.281)	1.01 (0.63–1.62)	1.09 (0.66–1.79)
	NK	183	64	(35.0%)	0.430 (0.336–0.551)	2.33 (1.66–3.27)	2.29 (1.60–3.28)

* Adjusting for year, country and quarter. CI = confidence interval.

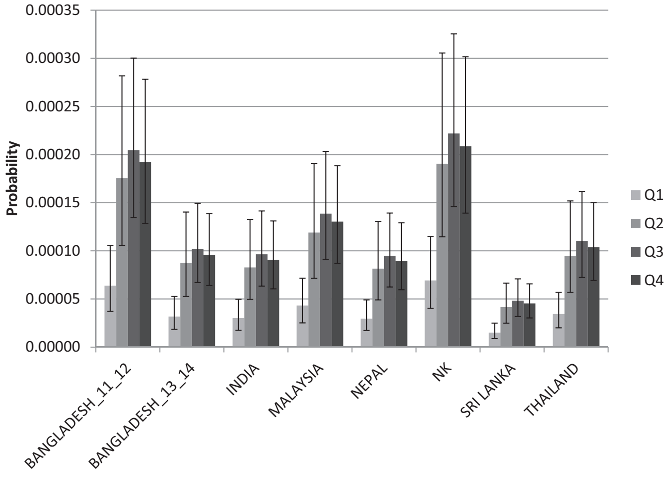

Fig. 2. Estimated probability of salmonellosis following consumption of one betel leaf by quarter and country using a dose response model. Q1 = January to March; Q2 = April to June; Q3 = July to September; Q4 = October to December. Confidence intervals represent a combined uncertainty from the 95% confidence interval for the MPN and the lower risk (from the 2.5th percentile) and the upper risk (from the 97.5th percentile) estimates from the dose response model.

probability of a betel leaf from India having at least one *Salmonella* varied between 0.017 (Q1) and 0.054 (Q3). The probability of acquiring infection following a single exposure of Indian leaves varied between 0.00003 (range 0.00002 to 0.00005) for quarter 1, and 0.00010 (range 0.00006 to 0.00014) for quarter 3.

To obtain numbers of cases occurring in the community and a risk to the population, the following assumptions were used: consumption was only considered within British Asian Indian, Pakistani and Bangladeshi for individuals, consumption only occurred in individuals over 30 years of age and a serving constituted eating a single leaf. Two scenarios were modelled: scenario 1, two servings per year for men and one per year for women; or scenario 2, 12 servings per year for men and six per year for women. Population estimates of British Asian Indian, Pakistani and Bangladeshi for individuals over 30 years of age in England in 2011 were 718,509 men and 696,644 women (Office of National Statistics, ONS, DC2101EW). Using the consumption estimates, this equates to 2.1 million servings per year for scenario 1 and 12.8 million servings per year for scenario 2. Based on the quarterly probability of infection (it was assumed that the servings were equally distributed throughout the year), the annual totals of salmonellosis cases were estimated using an exposure of *Salmonella* with a reduction in the numbers of *Salmonella* per leaf by 32% from the point of import to the point of retail sale and combined uncertainties were included based on both the dose response and the most probable number calculations. This resulted in estimates of 160 cases per year (range 102 to 242) for scenario 1 and 960 cases per year (range 612 to 1456) for scenario 2. For the numbers of cases reported to national surveillance, the estimates given above for the salmonellosis cases occurring in the community were divided by 4.7 and this equated to 34 cases per year (range 22 to 51) for scenario 1 and 204 cases per year (range 130 to 310) for scenario 2.

3.4. Characterisation of Salmonella

Of the 455 samples collected at the BIPs where *Salmonella* was detected, cultures from 442 (97%) were sent for further characterisation: at least one culture of *Salmonella* was sent from 207 (97%) of the 214 contaminated consignments. A total of 46 different serovars within *Salmonella enterica* subsp. *enterica* (subsp I) were identified in addition to 18 unnamed serovars, one *Salmonella enterica* subsp. *diarizonae* (subsp IIIb) and one *Salmonella enterica* subsp. *salamae* (subsp II). When considering representative cultures, 17% were *S.* Virchow; 10% *S.* Java; 7% *S.* Brunei; 7% *S.* Litchfield; and 6% *S.* Newport: 8% were different *Salmonella enterica* un-named serovars (Table 5).

Amongst the 214 contaminated consignments, between one and 13 *Salmonella* isolates were submitted for characterisation per consignment. Where 2 or more cultures were submitted, the same serovar was recovered from 24 (21%) samples on more than one occasion, and of the remaining 95 (79%) samples between 2 and 8 different serovars or more than one *Salmonella* subspecies were identified per consignment. Consequently, of all the 214 contaminated consignments, 95 (44%) samples had two or more *Salmonella* serovars detected. Amongst the 332 representative isolates from samples collected at the BIPs, the distributions of serovars per country of origin is shown in Table 5. The numbers of different *S. enterica* serovars from an individual country of origin detected from samples collected at BIPs were: 32 serovars from Bangladesh; 17 from India; 10 from Thailand; 4 from Malaysia; and one each from Sri Lanka or Nepal (Table 5).

Thirty four representative *Salmonella* isolates were selected from the 33 contaminated samples collected at wholesale, catering and retail, and 12 different *Salmonella enterica* subsp. *enterica* serovars (plus two unnamed serovars) were identified (Table 6). Amongst the representative cultures, 29% were *S.* Typhimurium, 18% *S.* Bareilly, and 15% *S.* Newport. The distribution of *Salmonella* serovars by different countries are shown in Table 6. Nine different serovars were detected from samples originating from Bangladesh, three from India and 2 serovars from Sri Lanka.

Amongst all the *Salmonella* submitted to GBRU (and not just the representative isolates) 74 were subjected to WGS and characterised according to their eBGs and ST. SNP addresses were assigned to 47 (64%) as they fell into common eBGs. Fifty seven of the *Salmonella* cultures were isolated from samples collected at BIPs and 17 collected at retail, wholesale or catering: 28 (38%) of the 74 cultures were isolated between 2011 and 2013 and the remaining 46 (62%) between 2014 and 2017. To further investigate the diversity within an individual *Salmonella* serovar, amongst the 74 isolates subject to WGS, single nucleotide polymorphism analysis and hierarchical single linkage clustering information was available for 8 isolates of *S.* Bareilly, 8 isolates of *S.* Newport, 17 isolates of *S.* Virchow, and 2 isolates of *S.* Weltevreden. Amongst these 35 isolates, 24 were collected at BIPs and 11 from retail. To illustrate the diversity detected, results for the 8 isolates of *S.* Bareilly and *S.* Newport are shown in Table 7. This Table also includes the only two isolates from human clinical diagnostic samples which were < 5 SNP differences from a betel leaf isolate. All pairwise comparisons of this dataset were 50 SNPs or greater for samples collected from different consignments at BIPs expect for two cultures of *S.* Virchow of identical SNP type which were isolated from separate samples collected from different consignments but collected on the same day at the same BIP and both with a country of origin of India. For the 11 samples from retail, all pairwise comparisons were 100 SNPs or greater for different sampling settings except for three cultures of *S.* Bareilly at the 25 SNP level (two from Bangladesh) independently sampled in 2015 and 2017 and three samples collected from the same shop on the same day where three isolates of *S.* Newport were detected, all with < 5 SNP differences (Table 7).

3.5. Comparison of characterisation of Salmonella from clinical diagnostic specimens with those recovered from betel leaves

Amongst all the 63,691 reported laboratory confirmed cases of salmonellosis in England during 2010 to 2017 where isolates were submitted to GBRU for characterisation, 41,348 (65%) were one of ten most common *S. enterica* subsp. *enterica* serovars, namely (in numerical order with the most numbers of cases first), *S.* Enteritidis, *S.* Typhimurium, *S.* Newport, *S.* Infantis, *S.* Virchov, *S.* Kentucky, *S.* Agona, *S.* Stanley, *S.* Java, and *S.* Bareilly. When considering the 366 representative isolates recovered from betel leaves (332 sampled at the

Table 5
Salmonella enterica subspecies and serovars detected and countries of origin from samples collected at Border Inspection Posts: 2011–2017: one representative isolate per serovar per consignment only included.

Salmonella enterica subspecies and serovars	Total	Numbers of consignments by countries of origin						
		Bangladesh	India	Malaysia	Nepal	Sri Lanka	Thailand	Not stated
Salmonella Agona	1		1					
Salmonella Augustenborg	14	9	2				1	2
Salmonella Bangkok	1	1						
Salmonella Bareilly	14	6	4					4
Salmonella Braenderup	2	2						
Salmonella Brunei	24	19					1	4
Salmonella Charity	5	4						1
Salmonella Chester	1	1						
Salmonella Chicago	1						1	
Salmonella Chichiri	1	1						
Salmonella Chittagong	1							1
Salmonella Deversoir	1		1					
Salmonella Eastbourne	6	5						1
Salmonella Enteritidis	2	1		1				
Salmonella Gaminara	4	4						
Salmonella Giza	1	1						
Salmonella Haifa	1		1					
Salmonella Houten	4	3						1
Salmonella Hvittingfoss	7	2	2				3	
Salmonella Infantis	4	1	1					2
Salmonella Java	34	25	5		1		1	2
Salmonella Javiana	4	4						
Salmonella Kentucky	3	2						1
Salmonella Kirkee	1		1					
Salmonella Lagos	1	1						
Salmonella Litchfield	22	19						3
Salmonella Matopeni	3	1	2					
Salmonella Montevideo	1	1						
Salmonella Newport	21	10	5	1		2	2	1
Salmonella Ohio	1							1
Salmonella Onireke	1	1						
Salmonella Poona	1							1
Salmonella Rubislaw	16	14						2
Salmonella Saint-Paul	1		1					
Salmonella Stanley	14	12						2
Salmonella Tananarive	1	1						
Salmonella Thompson	1						1	
Salmonella Typhimurium	14	9	4					1
Salmonella Uganda	1						1	
Salmonella Virchow	57	37	6	2			1	11
Salmonella Wangata	1	1						
Salmonella Weltevreden	12		6	2			4	
Salmonella Westminster	1	1						
Salmonella Weston	3	1						2
Salmonella Wilmington	1		1					
Salmonella Worb	2		2					
Salmonella enterica (un-named)[1]	27	20	5					2
Salmonella enterica subsp. *diarizonae* (61:l, v:1, 5, 7)	1		1					
Salmonella enterica subsp *salamae* (43:z4, z23:–)	1						1	1
Totals	332	216	48	4	1	2	17	44

[1] 27 *S. enterica* cultures with the following antigenic formulae: (1, 6, 14:d:–); (11:r:–); (13, 23:z:–); (17:d:–); (4, 5, 12:–:1, 2); (4, 5, 12:d:–); (6, 14:d:–); (6, 7:–:1, 2); (6, 7:y:–); (6, 8:i:–); (8, 20:y:–); (9, 12:–:1, 5); (9, 12:l, w:–); (9, 12:l, z28:–); (9, 12:l, –:1, 5); (9, 46:l, v:–); (I 6, 7:–:1, 2); (Rough:–:1, 2).

BIPs and 34 from retail), 164 (49%) and 24 (71%) were of the same serovars as the ten most common serovars causing human infection.

During 2011 to 2013, in the absence of the more enhanced comparative power provided by WGS, a comparison between isolates from human cases of salmonellosis and betel leave isolates were undertaken using the typing techniques available at that time (phenotypic serotyping, phage typing, antimicrobial resistance markers). The comparison did not identify any associations between human salmonellosis and betel leaf-consumption and no outbreaks linked or putatively linked to betel leaves were detected.

For the period 2014 to 2017 when characterisation by SNP analysis for common eBGs was implemented for all human clinical diagnostic and food isolates submitted to GBRU, no clusters were detected linked to consumption of betel leaves. However there were two sporadic human cases with isolates matching at ≤5 SNP level with betel leaf isolates (Table 7). The first was a *S.* Bareilly (eBG 206.2, ST203) isolated from the faeces of a 74 year-old female with a specimen collection date of August 2015 that was indistinguishable (i.e. matched at the 0-SNP level) to an isolate from betel leaves sampled in June 2015. The case lived in an urban area in the North West of England and name ontology analysis suggested a likely ethic origin of the Indian subcontinent. Exposure information with respect to betel leaf consumption was not available. The sample of betel leaves (of unknown country of origin) was collected at retail in July 2015 in North West of England less than 18 miles away from the home address of the case.

The second case where the patient's *Salmonella* isolate was at the ≤5 SNP threshold to that from an isolate recovered from a betel leaf, was due to *S.* Newport (eBG 7, ST 31) which was isolated from the faeces of

Table 6
Salmonella enterica serovars detected and countries of origin from samples collected at retail, wholesale or catering: 2011-2017: only one representative isolate per serovar per sample has been included.

Salmonella enterica subsp. *enterica* serovars	Total	Numbers of samples by countries of origin			
		Bangladesh	India	Sri Lanka	Not stated
Salmonella Augustenborg	1	1			
Salmonella Bareilly	6	4	1		1
Salmonella Brunei	1				1
Salmonella Hvittingfoss	1				1
Salmonella Litchfield	2	1		1	
Salmonella Newport	5	2	2		1
Salmonella Rubislaw	1	1			
Salmonella Typhimurium	10	6	2	2	
Salmonella Virchow	3	3			
Salmonella Weltevreden	3	1			
Salmonella Westminster	1	1			
Samonella enterica (4, 5, 12:d:–)		1			
Salmonella Unnamed (I Rough:z:–)	1	1			
Totals	34	22	5	3	4

a 44 year old male of East Asian origin in October 2017, who resided in an urban area of England. Exposure information with respect to betel leaf consumption was not available. The betel leaf was from Bangladesh which was on retail sale in July 2017 in a different region of England.

4. Discussion

Public health risks associated with consumption of foods of non-animal origin are becoming increasingly recognised (EFSA, 2013) and amongst all foods examined by Public Health England between 2011 and 2017, betel leaves were the most commonly *Salmonella*-contaminated ready-to-eat food tested (McLauchlin et al., 2018). We report here data on the *Salmonella* contamination of ready-to-eat betel leaves collected at the point of import and at retail, model the public health risk and describe an analysis of the data based on characterising the bacterium (including by WGS) which provides evidence for links to human salmonellosis in England.

We previously reported *Salmonella* contamination of betel leaves at retail (McLauchlin et al., 2018; Willis et al., 2015) and the results of these studies have been combined with further retail testing as well as those from betel leaf collected at BIPs. The contamination of samples collected at the point of importation indicates that contamination occurs in the countries of origin, although it does not indicate how this occurred. There are limited data available elsewhere on the microbiological quality of betel leaves for human consumption in their countries of origin, although studies from both India and Bangladesh have also reported *Salmonella* contamination of the betel leaves in these countries (Fakruddin et al., 2017; Haque et al., 2017; Nahar et al., 2018; Singh et al., 2006). The observation described here that the contamination rates were significantly greater in the third quarter of the year in this study may be associated with changes in water quality as a result of the South Asian Monsoon which occurs between June and September or to horticultural practices. The lack of testing during some periods of this study did not allow it to be established whether this effect occurs in all years and this would require further investigation. The exact routes of contamination in the countries of origin are not known and could occur during cultivation, particularly from contaminated water or after harvest for example via washing with contaminated water or from food handlers at any point in the food chain. Singh et al. (2006) reported the presence of *Salmonella* in water used at the retail level to drench betel leaves in India. Fakruddin et al. (2017) reported the possibility that *Salmonella* can become internalised within betel leaf tissue. Hence washing (including sanitisation) treatments are unlikely to remove all *Salmonella* from this product. There is therefore a possibility of cross-contamination of *Salmonella* from betel leaves to other products at retail, particularly since these products are often sold as loose in retail establishments (McLauchlin et al., 2018). We report here a significant reduction in the levels of contamination between the point of import at the BIP and at retail sale. It is not clear as to the reason for this but these may be due to washing steps applied to this product prior to sale or the die-off of the *Salmonella* due to drying on the leaf surface or natural plant constituents.

The *Salmonella* serovars detected (by both traditional and WGS approaches) in imported betel leaves described here were diverse and there were no associations detected with individual countries of origin; indeed almost all *Salmonella* isolates from different consignments appeared distinct and unrelated. This heterogeneous contamination

Table 7
Diversity demonstrated by WGS of *S.* Bareilly and *S.* Newport cultures isolated from betel leaves and matching isolates from the faeces of two patients with salmonellosis.

Country of origin	Setting	eBG	ST	SNP address	Relationship
S. Bareilly					
India	Retail 2017	206	909	1.1.63.97.97.98.98	
Not stated	Retail 2015	206.2	203	1.1.2.2.2.2.2	Three isolates from the same sample.
		206.2	203	1.1.2.2.2.2.2	
		206.2	203	1.1.2.2.2.2.2	
NA, patient with salmonellosis	Faeces 2015	206.2	203	1.1.2.2.2.2.2	< 5 SNP from three isolates from betel leaves above
Bangladesh	Retail 2017	206.2	203	1.1.2.110.111.114.118	
Bangladesh	Retail 2017	206.2	203	1.1.2.113.114.117.121	
Not stated	BIP 2015	206	909	1.1.54.84.84.85.85	Isolates from two independent samples collected from the same consignment
		206	909	1.1.54.84.84.85.85	
S. Newport					
India	Retail 2017	7	31	3.3.40.209.217.220.229	Three independent samples collected from same shop on same day
India	Retail 2017	7	31	3.3.40.209.217.220.228	
Not specified	Retail 2017	7	31	3.3.40.209.217.220.227	
Bangladesh	Retail 2017	7	31	5.5.5.212.221.224.233	
Sri Lanka	BIP 2016	3	46	43.97.119.133.141.148.164	Three isolates from the same sample
		3	46	43.97.119.133.141.148.165	
		3	46	43.97.119.133.141.148.164	
Bangladesh	Retail 2017	7	31	182.194.200.213.222.225.234	
NA, patient with salmonellosis	Faeces 2017	7	31	182.194.200.213.222.225.249	< 5 SNP from the isolate from betel leaves above

BIP = Border Inspection Post; eBG = e-Burst Group; NA = not applicable; SNP = single nucleotide polymorphism; ST = multilocus sequence type; WGS = whole genome sequencing.

indicates either contact with an inherently grossly contaminated source such as surface water contaminated with human or animal faeces, or a diverse range of sources of contamination through the food chain. Just over half (51%) of the serovars recovered from betel leaves occurred in the same ten serovars which were most commonly associated with human infections in England during the period of sampling. Hence this justifies further analysis of the data and investigation of the public health risks.

One tool to investigate the risks posed by *Salmonella* contamination of these types of products for communicable disease epidemiology, prevention and control is the ability to compare isolates derived from foods with those recovered from patients with enteric infections. Co-ordinated studies and microbiological monitoring of foods have been performed in England by PHE (and its predecessor organisations) for more than 30 years and have a risk-based rationale for examination of the specific food types or areas in the food chain. Surveys target specific food products and the results from testing provide valuable information on generic public health hazards in the food chain (Elviss et al., 2009; McLauchlin et al., 2018; Willis et al., 2015). Despite the low probability of foods associated with transmission of disease being sampled independently of outbreak investigations, co-ordinated surveys of food have identified hitherto undetected food vehicles (salad, fresh herbs and watermelon) associated with outbreaks of salmonellosis (Byrne et al., 2014; Elviss et al., 2009; Pezzoli et al., 2008; Sagoo et al., 2003). Consequently the analysis of *Salmonella* isolates from food has provided a unique source of data for communicable disease risk assessment, epidemiological studies and disease, prevention and control of outbreaks. However, the previous generation of typing tools were less discriminatory than WGS and is consequently less likely to identify associations both with small outbreaks or with sporadic cases of salmonellosis.

In this study we used the high discriminatory power of single nucleotide polymorphism (SNP) analysis of WGS data using a hierarchical single linked clustering method (Dallman et al., 2018) which indicated that while no outbreaks of salmonellosis were detected, two patients were identified where the clinical isolates of *Salmonella* were ≤ 5 SNPs from those recovered from samples of betel leaves. Case demographic data were consistent with a link to betel leaf consumption and in one instance the isolation from the betel leaf and from the patient clinical diagnostic sample were related temporally.

The close genetic match between the human cases and food isolates determined through WGS provides confidence of an association in both, presumably independent, instances, as the degree of genetic similarity seen between the isolates is considered indicative of a common source of contamination (Waldram et al., 2018a). However, routes of contamination for *Salmonella* are complex. For example, since these cases were associated with isolates taken at the retail level, it is possible that contamination could have occurred anywhere in the food chain from reservoirs in the countries of origin to a food handler prior to the point of consumption in England. After the end of this study in 2017 a further *S*. Bareilly was recovered from a betel leaf sample (country of origin Malaysia) sampled at a BIP which was ≤ 5 SNPs from that recovered from the 2015 patient and betel leaf sampled at retail further supporting a link to the patient from consumption of this food and *Salmonella* from this country of origin.

We report here, the use of a most probable number technique to provide quantitative data on *Salmonella* contamination. This approach used the replicate testing of consignments generated from routinely collected monitoring data for *Salmonella* in imported foods and allowed quantitative estimates of the bacterium at consumption. The probability of infection from consumption of betel leaves both to an individual consumer, and to the population. For the population estimate, it was not possible to obtain quantitative data on the amount of betel leaves imported into England, however we estimate in excess of 25 million leaves are imported through one of the BIPS in 2018. We have no information on the quantities coming from other routes (including those from other Member States within the EU) or clandestine imports which have been previously reported (McLauchlin et al., 2018). However even if import data were available, this will be problematic in terms of estimating the amount of betel leaves consumed since not all leaves are eaten and some are used for ritual, decoration as well as topical medicinal purposes. Furthermore, there are uncertainties about consumption practices including how the leaves are stored and handled, the percentage of the population in England that currently consume these leaves and the specific population characteristics that may influence vulnerability to infection and healthcare seeking behaviour. For the modelling described here, we therefore used a population based estimate from census data for British Asians (Indian, Pakistani and Bangladeshi) over 30 years of age in England in 2011. The two scenarios were modelled based on estimated numbers of servings which generate annual totals of 160 and 960 cases of salmonellosis in England. These scenarios may conservatively estimate consumption of betel leaves, not only because of the numbers of leaves passing through one of the BIPS (see above), but since only populations originating from three mainland countries in the Indian subcontinent were considered here: betel leaf is consumed in ethnic groups from Asian countries other than Bangladesh, India and Pakistan. There are uncertainties with the consumption estimates, however the numbers of servings are consistent with, albeit incomplete, data derived from: horticultural land use (an annual yield form one hectare in India is 6–7 million betel leaves; https://www.agrifarming.in/betel-leaf-farming-information/); the likely financial size of the market as €40 million to Bangladesh alone (Food Law Latest, 2016); and the more than 50 businesses registered as paan, pan or betel shops in the food hygiene rating scheme (http://ratings.food.gov.uk/). The numbers of cases estimated by the model may be an over-estimate since the dose response model (World Health Organization, 2002) is based on *Salmonella* from foodborne outbreaks and those from the environment may have a lower potential to cause human disease. The estimates of the numbers of cases may also be an over-estimate because these were modelled as independent events although individuals consuming betel levels are likely to do this on multiple occasions. Furthermore, there may be immunity to salmonellosis in individuals as a result of prior exposure to low levels of *Salmonella* through betel leaf consumption. Finally, the health seeking behaviour for betel leaf consumers may differ to that estimated for the total population by Tam et al. (2012) and this may over-estimate the numbers of cases diagnosed and reported to national surveillance. In the absence of better consumption and behavioural data, this simple population model was used to estimate exposure. Further work is needed to more accurately model exposures and better understand the public health burden with consumption of this product however this approach was designed to give an initial estimate the likely scale of the problem and generate hypotheses for further investigation.

In England, the majority of diagnosed cases of salmonellosis are reported to national surveillance and an isolates of *Salmonella* is submitted to the reference laboratory: all isolates submitted have been characterised by WGS since 2015. In this study, we modelled numbers of infections based on two different consumption patterns which estimated 34 and 204 cases of salmonellosis each year in England reported to national surveillance. It might therefore appear surprising that compared to the population estimates described here, only two human cases of salmonellosis in 2015 and 2017 were found where the patient's *Salmonella* isolates matched at the $<$ 5-SNP level with isolates from betel leaves. However, only 74 *Salmonella* isolates from betel leaves were tested here by WGS, only 64% of these were SNP typed, and the chances of identification of links will be very small because of the heterogeneity of the *Salmonella* contaminating these leaves. Because of this diversity, traditional approaches of analytical or descriptive epidemiology combined with strain typing will be unlikely to detect links between this food and clusters of infected patients.

We have further estimated that each consignment contains between 60,000 and 80,000 leaves. As a worst case scenario and assuming that a single consignment is contaminated at the levels detected from Bangladeshi products detected during 2011 and 2012. Using the model we describe here and taking into account the diversity of *Salmonella* both within and between consignments, we estimate that it would be unlikely that more than one case due to the same type would be reported to national surveillance. Consequently, since triggers detecting outbreaks in the current national surveillance systems are based on exceedances of a particular serovar or the detection of clusters at ≤5-SNP, then the recognition of clusters of cases associated with betel leaf consumption is unlikely to occur unless there is further secondary transmission such as within families, via consumption in a food handler, or if the bacterium is allowed to subsequently grow in a food item. Should the product become more widely consumed by the UK population, the risk will increase. There are ever-increasing demands for foods of Asian origin from a wider customer base, including using fresh leaves in different ways, such as flavourings for drinks and non-alcoholic beverages (Food Service India, 2017).

The data presented here allow some predictions on the likely epidemiological pattern of public health risks from betel leaf consumption. Firstly because of the consumption patterns, cases will be most likely to be confined to specific Asian ethnic groups, and are unlikely to occur in recognised outbreaks. Furthermore, cases will be due to a very diverse group of *Salmonella*, and will occur least often in the first three months of the year. We previously noted that a high proportion of betel leaf samples also contains high levels of indicator *Escherichia coli* (McLauchlin et al., 2018) and therefore contamination may not only be confined to *Salmonella*, but may include other enteric pathogens.

We suggest here that there are opportunities for improved communicable disease prevention and control by further integrating epidemiological and microbiological data from clinical samples with that from testing of foods. Data from food testing may facilitate elucidation of generic risk factors for disease (even in the absence of identification of links with infections) but can also be applicable to the investigation of 'sporadic' cases of salmonellosis (as well as other enteric infections). Ideally, there should be iterative processes to target food testing based on the results of hypothesis generation for food consumption habits as part of surveillance. Analysis of epidemiological, microbiological and behavioural data may lead to a better understand sporadic salmonellosis which should provide targets for interventions and improved source attribution for this infection. These types of analyses may also assist in the investigation of outbreaks caused by multiple strains of *Salmonella* (Hutchinson et al., 2018) as well as multiple enteric pathogens (Dallman et al., 2014; Waldram et al., 2018b). Efforts should be intensified to integrate WGS data originating from clinical isolates with those from food and the environment and should not only take place at the national level, but will provide valuable public health information when combined between countries.

Surveys and monitoring of foods can highlight, as in this report, where EU-wide legislative control measures are not fully effective. Risk managers have responded to the results of local testing and observation by implementing import controls at the EU level for betel leaves which has resulted in testing products at the point of import. More than 180 notifications as a result of the *Salmonella* contamination of betel leaves were generated between 2011 and 2017 through the Rapid Alert System for Food and Feed (RASFF), of which 96% were from the UK (https://webgate.ec.europa.eu/rasff-window/portal/?event=searchResultList&StartRow=201). In 2014 the EC implemented a temporary suspension of imported betel leaves originating or consigned by Bangladesh (Decision 2014/88/EU), which has now been extended to 2020 (Commission Implementing Decision 2018/935). Betel leaves from India and Thailand were subjected to an increased level of official control at the designated point of entry from March 2014 (Commission Implementing Regulation (EU) 323/214) and this continued for products from India in September 2015 (Commission Implementing Regulation (EU) 2015/1607). Control of betel leaves from India was extended in 2016, requiring that consignments be accompanied by results of sampling and analysis by the competent authorities in India for the absence of *Salmonella* (Commission Implementing Regulation (EU) 2016/166). It should be noted that the 70 *Salmonella* contaminated samples from India tested at BIPS all pre-dated the 2016 control requirement for consignments to be accompanied by the results of sampling and analysis (Commission Implementing Regulation (EU) 2016/166). All of the samples from Bangladesh collected from BIPs predate the 2014 EU Commission decision implementing a suspension of imports from this country (Decision 2014/88/EU). However samples on retail sale from both Bangladesh and India, and contaminated with *Salmonella,* were detected on retail sale in 2017 (McLauchlin et al., 2018).

Anecdotal evidence suggests that these types of food products can evade inspection at UK BIPs (including as undeclared or incorrectly labelled products) or are entering the EU through other Member States. We showed in this study that the contamination rates for betel leaves from Bangladesh in 2011–12 were significantly greater than those from other countries, and this supports the need for the greater controls applied to products from this country at this time. Further controls were applied to products from India and although there may be bias in the data presented here since more sampling took place as a result of checks targeting product from specific countries, *Salmonella* contamination rates were similar for products from other multiple Asian countries (including Bangladesh after 2012) and therefore country specific restrictions within the EU may not be effective in controlling importation of contaminated product. *Salmonella* contamination of any ready-to-eat food is recognised as a known hazard which should be further investigated and controlled.

In summary, we report here the consolidation of routine collected data from multiple sources to model the public health risks from the consumption of betel leaves. As with any model, the results should be interpreted with caution however the data presented here supports further investigation to estimate the burden of sporadic salmonellosis in England due to consumption of betel leaves to reduce the uncertainties around this assessment of risk. This food is consumed by specific ethnic groups and we predict that infections associated with betel leaf consumption will occur as sporadic infections which will not be recognised using the current surveillance strategies based largely on outbreak recognition. Should the product become more widely consumed by the UK population or used in different ways which will permit growth of the bacterium, the risk of infection will increase. However, *Salmonella* contamination of any ready-to-eat food is recognised as a known hazard which should be further investigated and effectively controlled.

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