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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

Pelczar JR, Harley JP, Klein DA (1993). Microbiology: Concepts and Applications. McGraw-Hill Inc., New York, pp. 591-603.

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Full Length Research Paper

Interaction of sex with age at diagnosis and radiation therapy in the survival of pediatric acute lymphoblastic leukemia

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Sex is a significant prognostic factor in the survival of pediatric acute lymphoblastic leukemia (ALL) with girls having superior outcome. This phenomenon could be partly due to the intrinsic relationship between sex and other prognostic factors. The present study aimed to assess the effect of sex on ALL survival after accounting for interactions of sex with age at diagnosis and radiation, in addition to known prognostic factors. We utilized 1973 to 2009 surveillance epidemiology and end results data. In a multivariable Cox proportional hazard model, stratified by the year of diagnosis, the prognostic value of sex diminished (adjusted hazard ratio (AHR) = 1.21, 95% confidence interval (CI): 0.93, 1.57). The difference in mortality between girls and boys was the lowest in the irradiated children diagnosed between ages 10 and 19 years. In this subgroup, boys' risk of mortality was not substantially different from that of girls (AHR = 0.96, 95% CI: 0.70, 1.33). In the large population based study, after accounting for the aforementioned interaction effects, the prognostic value of sex in ALL survival diminished, and it is eliminated in the irradiated children diagnosed between ages 10 to 19 years.

Key words: Sex, lymphoblastic leukemia, prognostic factors.

INTRODUCTION

Sex has consistently been shown to be associated with the prognosis and survival in pediatric acute lymphoblastic leukemia (ALL) (Carroll et al., 2003; Sather et al., 1981). Girls have a superior event-free survival compared to boys given equivalent therapy, whereas boys are at higher risk for genetic predispositions to ALL and treatment-related adverse events (Chessells et al., 1995; Dordelmann et al., 1999; Lanning et al., 1992; Nachman et al., 1998; Smith et al., 1996). Other clinical features that have been identified as having prognostic values include age at diagnosis, race, immunophenotype, chromosomal abnormalities and the number of primary tumor sites (Carroll et al., 2003; Hilden et al., 1995; Pui et al., 1994; Reaman et al., 1999; Rubnitz et al., 1997). Although pediatric ALL survival dramatically improved over the years because of advancements in early detection and treatment protocol, the survival difference by sex persists (Möricke et al., 2005; Reaman et al., 1985; Sather, 1986). This phenomenon could be due to the intrinsic relationship between sex and other prognostic

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prognostic factors. It has been reported that the proportion of boys diagnosed later in childhood is higher than that of girls, while increased age at diagnosis is associated with increased risk of mortality, and T-cell ALL, which has worse prognosis than B-cell ALL, occurs more commonly in boys, and those with T-cell ALL undergo more intensified therapies; on the other hand, the magnitude of the difference in ALL survival by sex is less apparent in recent studies with intensified therapy (Carroll et al., 2003; Möricke et al., 2008; Pui et al., 2009; Silverman et al., 2001).

Research on the relationship between the prognostic factors and their associations with pediatric ALL survival has been scanty, especially in the changing ALL survival and treatment protocols. The five-year survival rate of pediatric ALL patient was 43.2% in 1975 and 87% in 2005, and it has been found that many conventional risk factors have lost predictive strength (Pui, 1997). In this study, we sought to assess the extent of the association of sex and the survival patterns of pediatric ALL patients in the US, using the surveillance epidemiology and end result (SEER) data set from 1973 to 2009 after accounting for age at diagnosis, race, primary tumor sites, ALL immunophenotype, radiation therapy and interactions of sex with age at diagnosis and radiation therapy. In addition, we evaluated the impact of sex on the survival of irradiated ALL patients diagnosed between ages 10 to 19 years. The SEER program of the National Cancer Institute (NCI) is the most comprehensive and reliable source of population-based information in the United States on cancer incidence and survival (Ries et al., 1999). The SEER dataset is large and well representative to ALL patients in the US which is ideal for epidemiological investigation like ours. The patients were diagnosed during 1973 to 2009. Patients diagnosed in 1973 have as long as 37 years of follow-up time, while those diagnosed later had shorter periods of follow-up. In short, the survival patterns of pediatric ALL patients in the SEER dataset are not constant over the follow-up period. To account for this time-varying survival pattern, we stratified the analysis by the year of the diagnosis grouped into 5-year intervals.

MATERIALS AND METHODS

The study included 14192 children who were diagnosed with ALL between ages 0 to 19 years during 1973 to 2009, whose information was reported to one of the 17 SEER registries. The SEER data are estimated to represent 28% of the US population (Howlader et al., 2013; Ries et al., 1999). The SEER registries collect population data on age, sex, race, year of diagnosis, primary tumor sites, tumor morphology and follow-up for vital status (Howlader et al., 2013; Ries et al., 1999). The subgroup analysis included only children who were diagnosed between 10 and 19 years and received the radiation therapy. The data sets were accessed upon the approval of the SEER Data Access Agreement. The data were used only for the research purpose. All results were presented in a way that no individual could be identified. The study variables are described in brief.

Age at diagnosis

The SEER data included a variable of age at diagnosis recoded as < 1, 1 to 4, 5 to 9, 10 to 14 and 15 to 19 years. This age classification is representative of age based pediatric ALL risk groupings used in most studies and was adopted for the purpose of this paper.

Year of diagnosis

The study covered ALL patients diagnosed between 1973 and 2009. This variable was recorded in single-year interval. We recoded this variable into five-year intervals (1973 to 1977, 1978 to 1982, 1983 to 1987, 1988 to 1992, 1993 to 1997, 1998 to 2002, 2003 to 2007, 2008 to 2009). The interval 2008 to 2009 contains only two years. The maximum follow-up period of this study is 37 years.

Sex

Sex was a nominal variable in the SEER dataset and used as a binary variable with female as the reference group.

Race

In the SEER dataset, the variable race contained information of white, black, Asian/Pacific Islander and others or unknown. Because of the limited number of subjects, we did not include the later two categories in the analysis. We regrouped this variable as Caucasian (White), African American (Black) and Others/unknown (Asian/Pacific Islander, Others or Unknowns). African American (AA) was set as the reference group in our analyses.

Number of primary tumor sites

The minimum number of primary tumor site was 1 in the SEER data, while the maximum number was 3. In our preliminary exploration, we found 1.1% of patients with 2 primary sites and 0.1% with 3 primary tumor sites. Hence, we collapsed the variable into two categories: one primary and two or more primary sites. Patients with one primary tumor site were selected as the reference group.

ALL immunophenotype

The information on the ALL immunologic features was available in the SEER dataset. We used two distinct immunophenotypes: T-cell and B-cell/B-precursor. This variable was treated as binary, with the T-cell as the reference group.

Radiation therapy

Information of radiation therapy was listed as (a) Beam radiation, (b) combination of beam radiation with implant or isotopes, (c) none, (d) radiation, NOS, (e) recommended, (f) refused, and (g) unknown in the SEER dataset. The variable was dichotomized into “yes, had radiation therapy of any kind” versus “no, never had radiation therapy during the time of data collection”.

Follow-up time and survival status

The follow-up time was documented as the duration from the time of diagnosis to death from any cause or the last day of the available

survival information in the SEER registry. In the dataset, those who did not experience the event (death) during the follow-up time were censored. The survival status was determined on a binary scale, with 0 for censored and 1 for the event or failure.

Statistical analyses

Clinical features of ALL patients were summarized by sex. Categorical variables were described using frequencies and percentages, while continuous variables were summarized using medians, 75% percentiles, means and standard errors (SE). Pearson's χ^2statistic was used to examine the distribution of clinical features of ALL patients by sex. Five- and ten-year survival rates were calculated for boys and girls. The distribution of mortality was presented by sex, age at diagnosis, and the receipt of radiation. A univariable Cox proportional hazard model was performed to assess the effect of individual study variables. We utilized a multivariable Cox proportional hazard model, stratified by the year of diagnosis, to assess the extent of the association of sex with the survival of ALL after accounting for the effect of other influential factors found in the univariable model and interaction effects of sex with age at diagnosis and radiation therapy. In this regard, we performed two adjusted models with and without the inclusion of immunophenotype in the model. The variable immunophenotype had 5881 missing values. For both adjusted models, analyses were stratified by the year of diagnosis to account for the time varying survival in the follow-up period. In addition, we performed a subgroup analysis to compare the risk of mortality from ALL between girls and boys in irradiated children diagnosed between ages 10 to 19 years. The statistical software SAS version 9.3 (SAS institute, Cary, NC, USA) was used to perform the analyses.

RESULTS

There were 8,113 (57.2%) boys and 6,079 (42.8%) girls among 14,192 pediatric ALL patients in the SEER dataset. Table 1 presented the distributions of clinical and survival features of pediatric ALL by sex. There were no substantial differences in the distribution of girls and boys by race and year of diagnosis, $P > 0.05$. Compared to girls, more boys were diagnosed in each age at diagnosis group except that in the infants. The proportion of boys increased steadily with increased age of diagnosis (χ^2 for trend = 79.43, df = 1, $P < 0.0001$). Approximately, 48 and 68% of children diagnosed at < 1 and 15 to 19 years were boys, respectively. Marginally higher percentages of girls (1.6%) had multiple primary tumor sites compared to boys (1.1%), ($\chi^2 = 6.64$, df = 1, $P = 0.009$). There was a highly significant difference in the distribution of immunophenotype by sex ($\chi^2 = 127$, df = 1, $P < 0.0001$). Compared to girls (5.1%), a higher percentage of boys (10.4%) were found with T-cell phenotype. About 15.2% girls and 19.4% boys received radiation therapy. The 75th percentile survival time (SE) was 206 (22.55) months in girls and 74 (6.21) months in boys, while the mean survival time (SE) was 339.06 (2.88) months in girls and 323.67 (2.60) months in boys. The 5-year overall survival rate was 79%, while the 10-year survival rate was 74%. Girls had better 5 and 10-year survival rates (82 and 77%) compared to that of boys (77 and 71%). There was a significant difference in the distribution of deaths between boys and girls ($\chi^2 = 47$, df = 1, $P < 0.0001$), as all-cause-mortality was 19.2 and 24.0% in girls and boys, respectively. Table 2 displayed the distribution of mortality in pediatric ALL patients by sex, age at diagnosis and the receipt of radiation therapy. Although, the proportion of death was higher in boys than that in girls across all ages at diagnoses, this difference varied substantially depending on the receipt of the radiation therapy. Of the 449 children diagnosed at infancy, only 61 patients received radiation therapy of which 30 and 31 were girls and boys, respectively. The proportion of mortality was higher in boys (63.3%) than that in girls (48.3%) in this very young group of irradiated children, while there was no substantial difference in mortality between the non-irradiated girls (49%) and boys (50%) of the same age group of diagnosis.

Among the children diagnosed between 1 to 4 years of ages and received the radiation therapy, 26.3% of girls and 33.4% of boys died, while in the non-irradiated group, 9.9 and 13.0% of girls and boys died, respectively. Of those who were diagnosed at ages 5 to 9 years and received radiation therapy, 27.3% of girls and 34.0% of boys died, while only 14.8% of girls and 18.4% of boys died in non-irradiated patients of the same age at diagnosis group. Similarly, among children who were diagnosed between 10 to 14 years of age, 36.2 and 34.4% of irradiated girls and boys experienced death, respectively, while 25.2 and 29% of non-irradiated girls and boys died. In the children with age of diagnosis between 15 to 19 years, about 41.1 and 41.4% of irradiated girls and boys died, respectively, while 36.3 and 41.3% of non-irradiated girls and boys died, respectively.

Table 3 showed the factors associated with mortality in pediatric ALL in the SEER dataset. In this univariable Cox proportional hazard model, there was a highly significant difference in the mortality outcome in pediatric ALL by sexes. Boys were 1.29 times as likely as girls to die from ALL, hazard ratio (HR) = 1.29, 95% confidence interval (CI): 1.20, 1.39. Compared to the reference group, infants, the estimated HR of children diagnosed with ALL in the 1 to 4, 5 to 9, 10 to 14 and 15 to 19 age groups were HR = 0.18, 95% CI: 0.15, 0.20, HR = 0.26, 95% CI: 0.2,0.30, HR = 0.44, 95% CI: 0.38, 0.51 and HR = 0.69, 95% CI: 0.59, 0.80, respectively. AA pediatric ALL patients did differ from those of Caucasians with respect to mortality, and 44% were more likely to experience mortality, HR = 1.44, 95% CI: 1.27, 1.62. Although not significant, children diagnosed with multiple primary tumor sites showed a higher risk of death compared to those who had a single primary tumor site at the time of diagnosis, HR = 1.18, 95% CI: 0.92, 1.52. Children with the B-cell and B-precursor ALL had a lower hazard than those with T-cell ALL, HR = 0.57, 95% CI: 0.49, 0.65. Radiation, per se, did not improve survival; rather survival was worsened in patients received radiation therapy. Compared to the children who did not undergo radiation

Table 1. Characterization of Pediatric ALL Clinical Features by Sex, SEER Registry 1973-2009.

Variable	Female (%)	Male (%)	Chisq (df†)	*P*
Age group at diagnosis (years)				
<1	235 (3.9)	217 (2.7)		
1-4	2,830 (46.6)	3,553 (43.8)		
5-9	1,568 (25.8)	1,917 (23.6)	110.1 (4)	<0.0001
10-14	902 (14.8)	1,273 (15.7)		
15-19	544 (8.9)	1,153 (14.2)		
Race				
Caucasian	5,051 (83.1)	6,766 (83.4)		
African American	437 (7.2)	568 (7.0)	0.3 (2)	0.876
Other/Unknown	591 (9.7)	779 (9.6)		
Primary Tumor Site				
1	5,983 (98.4)	8,025 (98.9)	6.6 (1)	0.010
≥2	96 (1.6)	88 (1.1)		
Immunophenotype				
T-Cell	307 (5.1)	844 (10.4)	127.0 (1)	<0.0001
B-Cell, B-Precursor	3,174 (52.2)	3,986 (49.1)		
Radiation				
Yes	926 (15.2)	1,572 (19.4)	41.5 (1)	<0.0001
No	5,119 (84.2)	6,489 (80.0)		
Year of diagnosis				
1973-1977	313 (5.1)	439 (5.4)		
1978-1982	369 (6.1)	473 (5.8)		
1983-1987	402 (6.6)	536 (6.6)		
1988-1992	486 (8.0)	672 (8.3)	5.5 (7)	0.599
1993-1997	751 (12.4)	1,008 (12.4)		
1998-2002	1,368 (22.5)	1,727 (21.3)		
2003-2007	1,681 (27.7)	2,338 (28.8)		
2008-2009	709 (11.7)	920 (11.3)		
Survival time (months)				
75th percentile	206 (22.546)	74 (6.213)	-	-
Median	-	-	-	-
Mean (SE)	339.06 (2.88)	323.67 (2.60)	-	-
5-yr Survival	82.0	77.0	-	-
10-yr Survival	77.0	71.0	-	-
Mortality Status				
Dead	1,169(19.2)	1,951(24.0)	47.0 (1)	<0.0001
Alive	4,910 (80.8)	6,162 (76)		

†df: degrees of freedom.

therapy, the irradiated children had an approximately 1.66 times higher hazard, HR = 1.66, 95% CI: 1.53, 1.79, of dying from ALL. The HR decreased monotonically over the year of diagnosis, which implied the time-varying survival pattern during the follow-up period.

Table 4 presented the risk of mortality associated with

Table 2. Distribution of ALL patients (1973-2009 SEER Data) mortality by sex, age at diagnosis and receipt of radiation.

Age group at diagnosis (year)	Proportion of mortality			
	Without radiation		With radiation	
	Female (%)	Male (%)	Female (%)	Male (%)
<1	100 (49.0)	92 (50.0)	15 (48.4)	19 (63.3)
1-4	246 (9.9)	401 (13.0)	89 (26.3)	149 (33.4)
5-9	202 (14.8)	282 (18.4)	54 (27.3)	128 (34.0)
10-14	167 (25.2)	253 (29.0)	83 (36.2)	133 (34.4)
15-19	149 (36.3)	334 (41.3)	53 (41.1)	138 (41.4)

The number in parenthesis indicates the percentage of mortality in the corresponding group

Table 3. Hazard risk of mortality associated with sex and other prognostic factors in pediatric ALL patients (1973-2009 SEER data) in a univariable Cox proportional hazard model.

Variables	Hazard ratio (HR)	95% C.I.‡	*P*
Sex			
Female	1	-	Reference
Male	1.29	1.20, 1.39	<0.0001
Age at diagnosis (years)			
<1	1	-	Reference
1-4	0.18	0.15, 0.20	<0.0001
5-9	0.26	0.22, 0.30	<0.0001
10-14	0.44	0.38, 0.51	<0.0001
15-19	0.69	0.59, 0.80	<0.0001
Race			
Caucasian	1	-	Reference
AA	1.44	1.27, 1.62	<0.0001
Primary tumor sites			
1	1	-	Reference
≥2	1.18	0.92, 1.52	0.195
Immunophenotype			
T-Cell	1	-	Reference
B-Cell, B-Precursor	0.57	0.49, 0.65	<0.0001
Radiation			
No	1	-	Reference
Yes	1.66	1.53, 1.79	<0.0001
Year of diagnosis			
1973-1977	1	-	Reference
1978-1982	0.70	0.61, 0.80	<0.0001
1983-1987	0.56	0.49, 0.65	<0.0001
1988-1992	0.38	0.33, 0.44	<0.0001
1993-1997	0.32	0.28, 0.36	<0.0001
1998-2002	0.29	0.26, 0.33	<0.0001
2003-2007	0.24	0.21, 0.27	<0.0001
2008-2009	0.19	0.14, 0.25	<0.0001

‡C.I.: Confidence Interval.

Table 4. Hazard risk of mortality associated with sex in pediatric ALL patients (1973-2009 SEER data) in an adjusted multivariable Cox proportional hazard model, stratified by the year of diagnosis.

Variable	Model without immunophenotype			Model with immunophenotype		
	Adjusted hazard ratio (AHR)	95% C.I.‡	*P*	Adjusted hazard ratio (AHR)	95% C.I.	*P*
Sex						
Female	1	-	Reference	1	-	Reference
Male	1.21	0.93, 1.57	0.159	1.44	0.97, 2.14	0.069
Age at Diagnosis (year)						
<1	1	-	Reference	1	-	Reference
1-4	0.17	0.14, 0.21	<0.0001	0.13	0.09, 0.18	<0.0001
5-9	0.23	0.19, 0.29	<0.0001	0.22	0.15, 0.31	<0.0001
10-14	0.47	0.37, 0.58	<0.0001	0.42	0.29, 0.59	<0.0001
15-19	0.73	0.58, 0.93	0.009	0.70	0.49, 0.99	0.044
Race						
Caucasian	1	-	Reference	1	-	Reference
AA	1.45	1.28, 1.64	<0.0001	1.33	1.10, 1.61	0.004
Primary tumor sites						
1	1	-	Reference	1	-	Reference
≥2	1.17	0.90, 1.50	0.241	0.69	0.45, 1.07	0.094
Radiation						
No	1	-	Reference	1	-	Reference
Yes	0.90	0.78, 1.03	0.128	1.13	0.88, 1.45	0.330
Sex * age at diagnosis (year)						
Male* 1-4 years	1.09	0.82, 1.47	0.551	1.01	0.64, 1.60	0.961
Male* 5-9 years	1.12	0.82, 1.52	0.474	0.89	0.56, 1.41	0.618
Male* 10-14 years	0.90	0.66, 1.22	0.493	0.79	0.50, 1.25	0.315
Male * 15-19 years	0.92	0.68, 1.26	0.599	0.77	0.49, 1.22	0.271
Sex* radiation	1.07	0.91, 1.27	0.418	0.87	0.65, 1.17	0.346
Immunophenotype						
T-Cell	-	-	-	1	-	Reference
B-Cell, B-Precursor	-	-	-	0.84	0.73, 0.97	0.016

* indicates interaction. ‡C.I.: Confidence Interval

sex in pediatric ALL patients estimated from two multivariable variable Cox proportional hazard models. The first model was adjusted for the effects of age at diagnosis, race, number of primary tumors, radiation, interactions of sex with age at diagnosis and radiation. The second model included immunophenotype in addition to the variables in the first model. After accounting for the main and interaction effects in the first model and stratification by the year of diagnosis, the significance of the difference in survival between girls and boys, found in univariable model, failed to persist, adjusted HR (AHR) = 1.21, 95% CI: 0.93, 1.56, $P = 0.15$. After inclusion of immunophenotype in the model, the significance of the sex difference still failed to persist in a relatively smaller number of patients.

Table 5 illustrated the hazard risk of mortality associated with sex in irradiated pediatric ALL patients diagnosed between ages 10 and 19 years, estimated in two multivariable variable Cox proportional hazard models. The first model was adjusted for the effects age at diagnosis and race, and the other model included immunophenotype in addition to the age at diagnosis and race. There was no substantial sex difference in the risk of mortality in this subgroup of irradiated pediatric ALL

Table 5. Hazard risk of mortality associated with sex in pediatric ALL patients diagnosed between ages 10 and 19 years who received radiation therapy (1973-2009 SEER dataset) in an adjusted multivariable Cox proportional hazard model, stratified by the year of diagnosis.

Variable	Model without immunophenotype			Model with immunophenotype		
	Adjusted hazard ratio (AHR)	95% C.I.‡	*P*	Adjusted hazard ratio (AHR)	95% C.I.	*P*
Sex						
Female	1	-	Reference	1	-	Reference
Male	1.09	0.89, 1.35	0.41	0.96	0.70, 1.33	0.819
Age at diagnosis (year)						
10-14	1	-	Reference	1	-	Reference
15-19	1.33	1.10, 1.62	0.005	1.34	1.00, 1.81	0.052
Race						
Caucasian	1	-	Reference	1	-	Reference
AA	1.21	0.83, 1.76	0.32	1.24	0.73, 2.10	0.433
Immunophenotype						
T-Cell	-	-	-	1	-	Reference
B-Cell, B-Precursor	-	-	-	1.49	1.07, 2.08	0.018

‡C.I.: Confidence Interval

patients; AHR = 1.09, 95% CI: 0.89, 1.35 and AHR = 0.96, 95% CI: 0.70, 1.33 in the first and second models, respectively.

DISCUSSION

Sex disparity in the survival of pediatric ALL has long been consistently reported (Carroll et al., 2003; Pui et al., 1999; Sather et al., 1981). The present study have attempted to assess the impact of sex on ALL survival after accounting for the effects other known prognostic factors and their interactions with sex. There are few findings from our study. First, the distribution of mortality from pediatric ALL by sex is substantially different by the receipt of radiation therapy and age at diagnosis. Secondly, the significance of sex on the survival of pediatric ALL has diminished after accounting for the effects of the following prognostic factors: age at diagnosis, receipt of radiation therapy, race, immunophenotype and interactions of sex with age at diagnosis and receipt of radiation therapy. Thirdly, there is no substantial impact of sex on the survival of pediatric ALL among irradiated patients diagnosed between 10 and 19 years.

In the SEER population based dataset of pediatric ALL, difference in mortality by sex remained consistent over the age at diagnosis in non-irradiated children, except among those diagnosed during infancy (Table 2). The result of this disparity in pediatric ALL survival is supported by findings of the previous studies (Carroll et al., 2003; Chessells et al., 1995; Sather et al., 1981). No difference in mortality by sex has been observed in non-irradiated children diagnosed during infancy. However, among the irradiated children, substantially differential mortality patterns have been observed between girls and boys over the age at diagnosis. The irradiated children, diagnosed during infancy have shown the largest difference in mortality, 15%, between girls (48%) and boys (63%). In fact, the proportion of deaths in girls of this group of children is the same as that in non-irradiated children of the same age of diagnosis. Irradiated boys diagnosed between ages 1 to 9 years are likely to have a higher mortality compared to irradiated girls of the same ages. While, no difference in mortality is observed between irradiated girls and boys diagnosed between ages 10 to 19 years. Radiation therapy may likely to have a key role in the removal of the sex disparity of mortality in this group of pediatric ALL patients. This finding is partly supported by previous studies showing a lessened sex disparity in survival due to the use of intensified therapy (Carroll et al., 2003; Pui et al., 2009; Silverman et al., 2001).

Although boys have shown a highly significantly increased risk of mortality compared to girls in the un-adjusted analysis, the significance of this sex difference in the mortality has diminished after the adjustment for the prognostic factors of age at diagnosis, race, receipt of radiation therapy, interaction of sex with age at diagnosis, and the interaction of sex with the receipt of radiation therapy. In a subgroup analysis of irradiated children

diagnosed between ages 10 to 19 years, no substantial effect of sex on the survival of pediatric ALL is observed in a model adjusted for age at diagnosis and race (Table 5). This result is further enhanced with the inclusion immunophenotype in the model (Table 5).

In addition, our study has confirmed the prognostic value of age at diagnosis (Carroll et al., 2003; Möricke et al., 2005; Sather, 1986), immunophenotype (Pui et al., 1993) and race (Bhatia et al., 2002; Kadan-Lottick et al., 2003) which are identified as the important predictors of pediatric ALL survival in previous studies .

Like other epidemiological studies, the current study is not without limitation. First, our results may be driven in part by the effect of unmeasured confounders. There is no other treatment information except radiation therapy in the SEER data. Secondly, the follow-up periods tend to be shorter for children diagnosed more recently. However, our results are not limited by this variability of the follow-up time, since we have stratified the analysis by the year of diagnosis. Despite the limitations, epidemiologic studies often utilize population based large datasets and are able to provide naive insight into the etiology of the disease and treatment processes which are later verified through intensive investigations.

Conclusion

The sex disparity in the survival of pediatric ALL may be partly explained by its interaction with age at diagnosis and radiation treatment; and the radiation therapy could eliminate the sex disparity in the survival of ALL patients after diagnosed of 10 years. Further research is warranted to validate the findings of this study.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Impact of environmental factors on the prevalence of autistic disorder after 1979

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The aim of this study was to investigate a previously overlooked, universally introduced environmental factor, fetal and retroviral contaminants in childhood vaccines, absent prior to change points (CPs) in autistic disorder (AD) prevalence with subsequent dose-effect evidence and known pathologic mechanisms of action. Worldwide population based cohort study was used for the design of this study. The United States, Western Australia, United Kingdom and Denmark settings were used. All live born infants who later developed autistic disorder delivered after 1 January 1970, whose redacted vaccination and autistic disorder diagnosis information is publicly available in databases maintained by the US Federal Government, Western Australia, UK, and Denmark. The live births, grouped by father's age, were from the US and Australia. The children vaccinated with MMRII, Varicella and Hepatitis A vaccines varied from 19 to 35 months of age at the time of vaccination. Autistic disorder birth year change points were identified as 1980.9, 1988.4 and 1996 for the US, 1987 for UK, 1990.4 for Western Australia, and 1987.5 for Denmark. Change points in these countries corresponded to introduction of or increased doses of human fetal cell line-manufactured vaccines, while no relationship was found between paternal age or Diagnostic and Statistical Manual (DSM) revisions and autistic disorder diagnosis. Further, linear regression revealed that Varicella and Hepatitis A immunization coverage was significantly correlated to autistic disorder cases. R software was used to calculate change points. Autistic disorder change points years are coincident with introduction of vaccines manufactured using human fetal cell lines, containing fetal and retroviral contaminants, into childhood vaccine regimens. This pattern was repeated in the US, UK, Western Australia and Denmark. Thus, rising autistic disorder prevalence is directly related to vaccines manufactured utilizing human fetal cells. Increased paternal age and DSM revisions were not related to rising autistic disorder prevalence.

Key words: Autism disorder, change point, vaccine, paternal age.

INTRODUCTION

Autistic disorder (AD) is a subset of the Autism Spectrum Disorders (ASDs), a group of developmental disabilities that have reached epidemic levels. Worldwide, 1988 has been identified by the Environmental Protection Agency (EPA) as a critical incident year for AD (McDonald 2010). The CDC released a study in 2013 estimating US ASD

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prevalence at 1 in 50 children aged 6 to 17 in 2011 to 2012. In addition to ASD, there are also apparent epidemic levels of other early onset neuro-developmental (ND) syndromes such as childhood onset schizophrenia (0.4% of population affected) (Okkels et al., 2012) and bipolar disorder (Leibenluft 2008). Shared characteristics among childhood ND epidemics include associations with male gender, reduced reproductive fitness, increased paternal age and the presence of excess de novo genomic mutation rates. Paternal age is currently a favored explanation for the worldwide autism epidemic. However, evolving concepts about autism spectrum and other ND diseases suggest these diseases to be ‘‘multi-hit’’ with genetic, genomic and environmental contributors. Accumulating evidence from family-based exome sequencing points to the importance of hundreds of rare, diverse, de novo mutations (DNMs) inchildhood ND diseases (Van Den Bossche et al., 2012; Robinson 2010; Awadalla et al., 2010; O'Roak et al., 2011; De Ligt et al., 2012; Girard et al., 2011; Xu et al., 2012).

The de novo mutations in these diseases are consistently found in exons or critical coding regions of genes that would lead to premature stopornon-functional proteins (Awadalla et al., 2010; O'Roak et al., 2011; De Ligt et al., 2012; Luo et al., 2012). In addition to the increase in DNMs in children with ND disease, de novo genomic insertions and deletions are significantly increased in intellectual disability (De Ligt et al., 2012), autistic disorder (O'Roak et al., 2011), and childhood onset schizophrenia (Xu et al., 2012). Diverse, rare DNMs mandate that environmental factors known to cause genomic instability be evaluated for their relationship to these diseases.

Consideration of potential environmental triggers requires statistical assessment to identify birth year change points (CPs) associated with a rising rate in the incidence of autism. Requirements for an environmental factor as a trigger for disease include: (1) absent or lower levels before a change point, (2) continued increase after a change point is demonstrated (dose-effect), (3) biological mechanism consistent with pathology, and (4) in instances of non-geographically limited disease such as autism, schizophrenia and intellectual disability, it should have almost universal exposure (McDonald 2010). This study investigates a previously overlooked, universally introduced environmental factor, fetal and retroviral contaminants in childhood vaccines, absent prior to change points in autistic disorder prevalence with subsequent dose-effect evidence and known pathologic mechanisms of action. Vaccinations have done tremendous good in the world; however, further investigation of fetal manufactured-vaccine contaminants as an environmental contributor to the current autistic disorder epidemic is called for.

METHODOLOGY

Previously published autistic disorder data obtained from large populations and having a time span adequate for change point analyses were used. For the Diagnostic and Statistical Manual (DSM) revisions, change points are predicted based on the year of FDA approval of the vaccine and the month or year of publication of the DSM revision, respectively, and compared to the statistically calculated autistic disorder change points.

Data sources

Broadening changes in diagnostic criteria for ASD complicate interpretation of the current epidemic. Therefore, we focused on autistic disorder (previously called infantile autism), the most severe form of ASD, which has relatively constant diagnostic criteria over the past 5 decades, despite nomenclature changes from childhood schizophrenia to infantile autism to autistic disorder (McDonald 2010). To objectively assess suspected diagnostic relaxation for autistic disorder, printing dates were obtained for the DSM editions, found on the copyright page. Printing dates indicate the rapidity with which changes in diagnostic criterion were disseminated to the professional community. To determine whether DSM revisions were related to autistic disorder, we predicted a range of expected autistic disorder change point birth years based on the printing dates for the various DSM revisions. If DSM revisions cause an autistic disorder change point, children born prior to the new edition would be affected. Expected change point ranges are predicted to be 8 years prior to the earliest printing date and 3 years prior to the latest printing date for each revision based on first diagnosis of AD occurring after 3 years of age and firm diagnosis by 8 years of age (Lord et al., 2006; Luyster et al., 2009).

For the US, autistic disorder data were obtained from the California Department of Developmental Services (DDS) (McDonald 2010; Cavagnaro 2003; Schechter and Grether 2008) and from the Individuals with Disabilities Education Act (IDEA) program website of the Department of Education (IDEA 2012). Live birth data were extracted from the CDC's "Annual reports of Vital Statistics of the United States", (Centers of Disease Control and Prevention 2012a; Centers of Disease Control and Prevention 2012b) and birth year autistic disorder prevalence per 10,000 was then calculated. Male population data were obtained from the U.S. Census Bureau website, (US Census Bureau 2012a) for data prior to 2000 and from the "fact finder" web site for data after 2000 (US Census Bureau 2012b). Birth rates by age of father were obtained from the National Vital Statistics Reports: "Birth Final Data" (Centers of Disease Control and Prevention 2012). Varicella and Hepatitis A immunization coverage for children 19 to 35 months of age was obtained from the CDC National Immunization Survey (NIS) (Centers of Disease Control and Prevention 2012).

For Western Australia, autistic disorder prevalence for children aged 2 to 3, 4 to 5 and 6 to 8 years was obtained from (Nassar et al. 2009). Live births, live births by paternal age cohort, and male population data were obtained from the Australian Bureau of Statistics (Australian Bureau of Statistics 2013). Childhood autistic disorder data for North East London and Denmark were from (Lingam et al., 2002; Lauritsen et al. 2004), respectively.

Linear regression and change point analysis

Linear regression and R^2 analyses were used to assess correlations between autistic disorder prevalence and vaccine coverage or births by paternal age; associations with $P<0.05$ were considered significant.

For change point determination, both the hockey-stick (Qian 2010) and segmented line fitting (Muggeo 2008) methods were employed. The robustness of our algorithm was tested by repeating the algorithm using deliberately chosen poor initial inputs. Our fit results were robust across a wide variation of input parameters (data

not shown).

The Akaike Information Criterion (AIC) (Sakamoto et al., 1986) and the Bayesian Information Criterion (BIC) (Tiwari et al., 2005) determined the optimal segmented line fits and associated change points. The R statistical software was used to run the 'segmented' and AIC algorithms. For the data presented, all possible pairs of input change point years were tested. All other input parameters were set to default values. Not all pairs of input years led to convergence; what are presented here are results from fits that converged and had the lowest AIC and BICs cores.

Cell substrate residuals in selected childhood vaccines

Residual human DNA (single and double stranded) levels from the human fetal cell lines used to manufacture Meruvax® (Rubella, Merck & Co. Inc.), the rubella component of MMRII®, and HAVRIX® (Hepatitis A, GSK Biologicals) were measured using commercially available ELISA kits (Pico Green (dsDNA) and OliGreen (ssDNA)) (Life Technologies). DNA fragment sizes were determined using SYBR gold staining after 4% agarose gel electrophoresis. Notably, the viruses in the Meruvax®, MMRII® and HAVRIX® vaccines are mRNA viruses, not DNA viruses, and since the mRNA was degraded by heat treatment prior to oligonucleotide measurements, the DNA results are indeed specific for human DNA, the only DNA in the mRNA virus vaccines (Oker-Blim et al., 1984; Wikipedia 2014a; b; c).

RESULTS

Autistic disorder change point analysis

Segmented line fitting analysis identified three changepoints from the US IDEA and CA DDS AD data for birth years 1970 to 2002; 1980.8 (Figure 1A; panels A and B), 1988.4 (panel B), and 1995.6; 1996.5 (panels C and D). Since hockey-stick analysis of IDEAAD data for 19-year-olds born during1973 to 1987 identified an autistic disorder change point at birth year 1980.8 (Figure 1A; panel A) which had not been published by the EPA (McDonald 2010), hockey-stick was compared to segmented line fit for California DDS data which had been used in the EPA publication for birth years 1970 to 1997 (Figure 1B). Based on the AIC and BIC, the segmented algorithm with 2 change points (1980.9 and 1988.4) resulted in a better fit of the data than the hockey-stick method used by the EPA, which identified a single change point at birth year 1987.5. When directly compared, our software program analysis to the EPAs, use of the hockey-stick method yielded a change point for the CA DDS data for birth years 1970 to 1997 equivalent to the EPA's published change point to the nearest tenth (Figure 1B).

The graph in Panel E depicts change points when all autistic disorder data from US IDEA and CA DDS for children born between 1973 and 2002 used in panels A through D are combined and submitted to segmented line fitting algorithms. Using the combined data, three change points are calculated (1980.8, 1988.4 and 1996.5) demonstrating the robustness of segmented line fitting for change point analysis. Panel F shows segmented line fit results for North East London (UK) for birth years 1979 to 1995 (core AD, CP: 1987). Panels G and H show results for Western Australia for birth years 1983 to 1999 (CP: 1990.4) and Denmark for birth years 1964 to 1995 (CP: 1987.5).

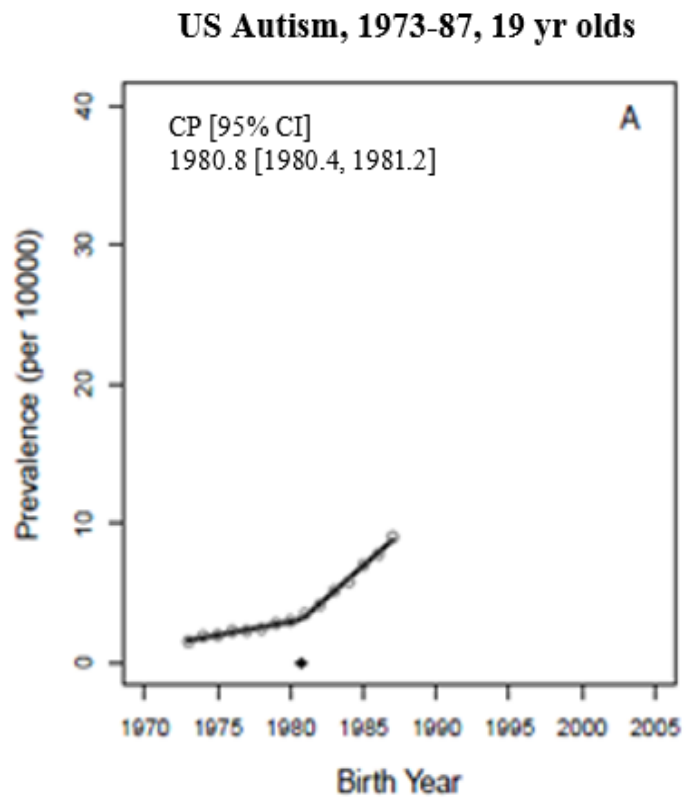
Diagnostic and statistical manual (DSM)

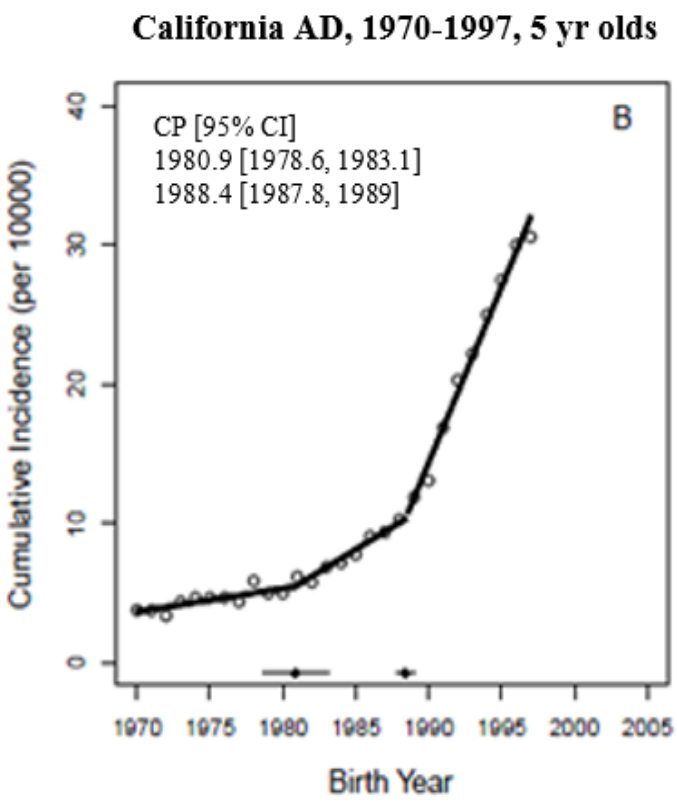
The first DSM of Mental Disorders, *DSM I*, was published by the American Psychiatric Association in 1952. Since then there have been five major revisions: *DSM II* (1968); *DSM III* (1980); *DSM III – R* (1987); *DSM IV* (1994) and *DSM IV – TR* (2000). The impact of DSM revisions on the diagnosis of autism depends on the significance of changes to diagnostic criteria and on the rapidity with which the DSM revisions are disseminated and applied. Table 1 compares diagnostic criteria for autistic disorder, but not the broader autism spectrum disorder, across DSM revisions. As the table demonstrates, DSM revisions differ primarily in that more examples of behaviors typical of autistic disorder were listed with each revision. However, the required number of behaviors for an autism diagnosis remains the same or actually increases with the revisions, rather than becoming less stringent as has been commonly suggested. Furthermore, if relaxed diagnosis were to lead to an increase in autistic disorder prevalence then one would expect a decrease in the number of symptom categories required for diagnosis, however, these symptom categories are consistent across DSM revisions.

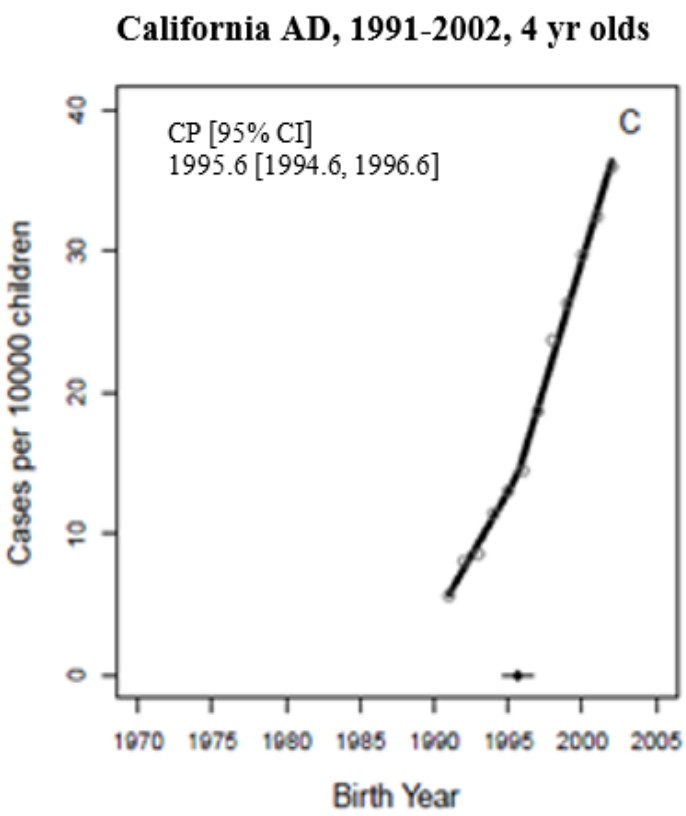
The DSM printing record (Table 2) suggests that the dissemination and application of the DSM revisions is quite rapid after the date of DSM publication, and therefore, the printing dates for DSM were used to predict expected birth year change points to determine whether DSM revisions affect autistic disorder diagnosis rates. Predicted expected birth year change point ranges are found in Table 2. Change point ranges are predicted to be 8 years prior to the earliest printing date and 3 years prior to the latest printing date for each revision based on first diagnosis of autistic disorder occurring after age 3 and firm diagnosis by age 8 (Lord et al., 2006; Luyster et al., 2009). Assuming that the DSMs are strictly followed, the latest predicted birth year change points as a result of DSM changes are 1978, 1984, and 1992 for DSM-III, IIIR, and IV, respectively. There is no corresponding calculated autistic disorder change points associated with those years (Table 2), therefore DSM revisions are unlikely to be the primary trigger for increased autistic disorder prevalence.

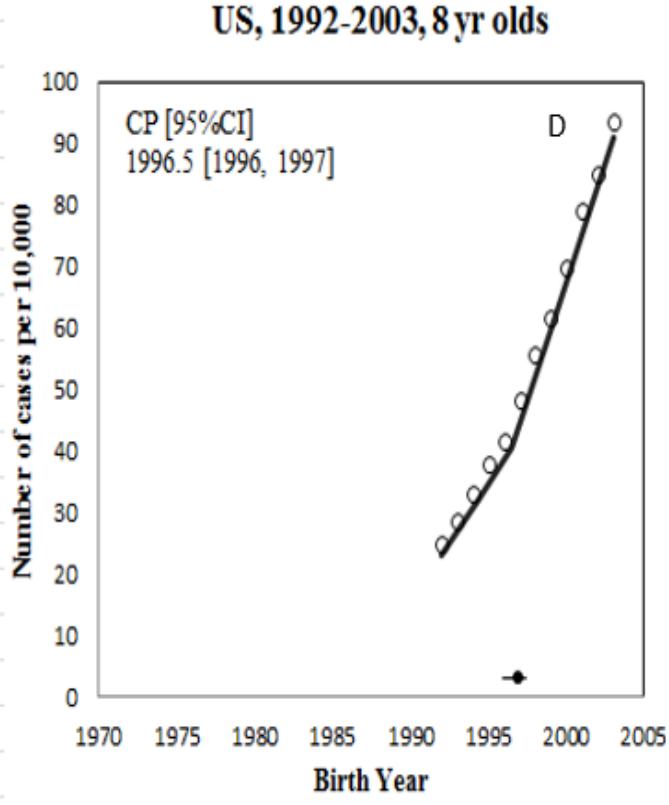
Association between paternal age and autistic disorder

Figure 2A shows that US live births declined during the 1960s and 1970s in almost all paternal age groups, and then rebounded after 1978 in all paternal age groups above


US Autism, 1973-87, 19 yr olds
CP [95% CI]
1980.8 [1980.4, 1981.2]
A
Prevalence (per 10000)
Birth Year


California AD, 1970-1997, 5 yr olds
CP [95% CI]
1980.9 [1978.6, 1983.1]
1988.4 [1987.8, 1989]
B
Cumulative Incidence (per 10000)
Birth Year


California AD, 1991-2002, 4 yr olds
CP [95% CI]
1995.6 [1994.6, 1996.6]
C
Cases per 10000 children
Birth Year


US, 1992-2003, 8 yr olds
CP [95%CI]
1996.5 [1996, 1997]
D
Number of cases per 10,000
Birth Year

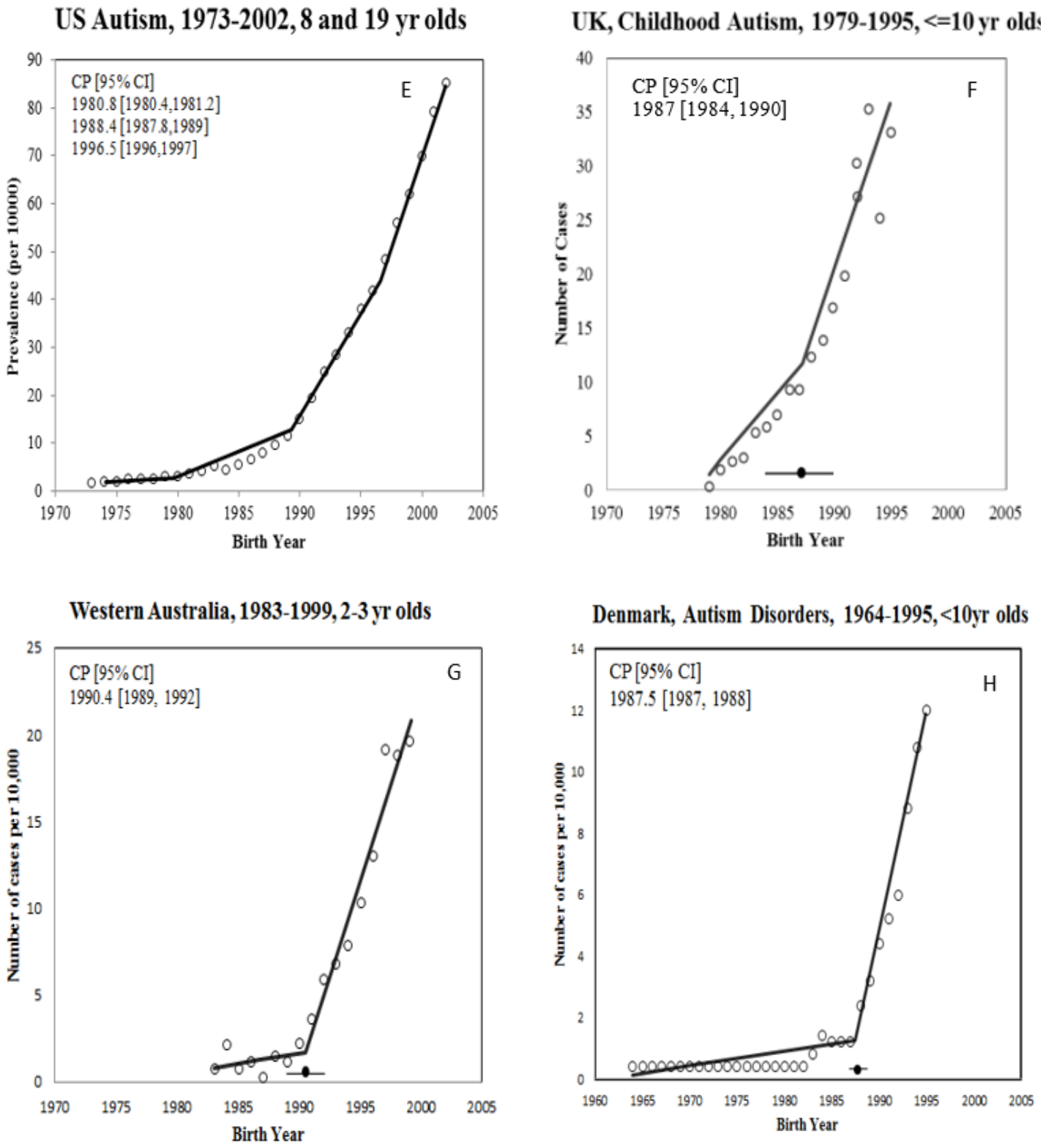


Figure 1. AD changepoint analysis robustness and results.
Figure 1A shows AD changepoint results for the U.S., California, UK, Western Australia, and Denmark. Figure 1B shows a comparison of 'hockey' and 'segmented' fits for California AD 1970-1997 data. Both analyses yield changepoints with overlapping confidence intervals near 1988. However, 'segmented' analysis reveals a second changepoint near 1981.

Table 1. Comparison of diagnostic criteria for autistic disorder (AD) across DSM revisions.

Autistic disorder symptom category	DSM II (1968) Schizophrenia, childhood type	DSM III (1980) Infantile autism	DSM III R (1987) Autistic disorder	DSM IV (1994) and DSM IV R (2000) Autistic disorder
	Number of symptom examples listed/Number of examples required for diagnosis			
Impaired social interaction e.g. pervasive lack of responsiveness to other people	3 examples/3 requirement not listed	1 example/1 required	5 examples/2 required	4 examples/2 required
Impaired communication e.g. marked abnormalities in the production of speech, including volume, pitch, stress, rate, rhythm, and intonation; stereotyped and repetitive use of language or idiosyncratic language.	1 example/1 required	4 examples/4 requirement not listed	6 examples/1 required	4 examples/1 required
Atypical or withdrawn behavior e.g. stereotyped body movements (for example, hand flicking or twisting, spinning, head-banging, complex whole-body movements	1 example/1 required	2 examples/2 requirement not listed	5 examples/1 required	4 examples/1 required
Age of onset	Before puberty	Before 30 months	Before 36 months unless specified	Before 36 months
Alternative diagnosis that must be excluded	Schizophrenia symptoms	None listed	None listed	Rett's disorder* or childhood disintegrative disorder

groups above the age of 30. Of note, fathers over the age of 40 had similar numbers of live births in 1963 (333,785) as they did in 2001 (342,030); therefore, if paternal age were a major trigger for autistic disorder, older fathers would have been fathering as many autistic children in 1963 as 2008. However, reported autistic disorder prevalence was 0.7 cases per 10,000 in 1963 (Treffert 1970) compared to 79 per 10,000 in 2001. In addition, linear regression analysis of paternal age versus autistic disorder for each specific birth year did not reveal a relationship (Figure 2B; R2 = 0.1027).

In Western Australia, from 1975 to 2011, live births increased slightly for fathers over 40 years of age (Figure 2C). However, live births in 1999 to fathers over age 40 were less than 2-fold higher than in 1989, while autistic disorder diagnosis had risen 10-fold between birth years 1989 and 1999. Linear regression analysis revealed no relationship between paternal age and autistic disorder diagnosis for Western Australia (Figure 2D; R^2<1).

Association between approval of human fetal cell line manufactured vaccines and autistic disorder change points

Published AD data for the UK (Taylor et al., 1999) and North East London (Lingam et al., 2002) suggested that autistic disorder rose conspicuously around 1988 to1989, and our calculated change point for the North East London data is 1987. While MMR coverage was over 90% before this time (Lingam et al., 2002), the autistic disorder change point followed a switch in the UK from animal cell line to human fetal cell line manufacture of MMR vaccine in October 1988 (Table 3). Similarly, our calculated change point result of 1987.5 for Denmark corresponds to the introduction of MMR vaccine in 1987. The relationship between autistic disorder prevalence and use of vaccines manufactured were therefore evaluated using human fetal cell lines elsewhere.

The US 1980 to 1981 autistic disorder change point followed the January 1979 approval of MeruvaxII® and MMRII®, which are manufactured in the human fetal cell line WI-38. An earlier human fetal cell vaccine, Diplovax, had been licensed in the US in 1972. However, it was withdrawn in 1976 because it never gained any US

Table 2. Printing schedules for DSM revisions/editions.

Parameter	Date of printing	Number printed	Predicted BYr CP range by DSM printings	Calculated BYr CP
DSM III	February-80	40,000	February 1972 - September 1978	1980.85*
	May-80	25,000		
	September-80	25,000		
	November-80	30,000		
	January-81	30,000		
	March-81	35,000		
	September-81	25,000		
DSM IIIR	May-87	75,000	May 1979 - November 1984	1988.4
	June-87	80,000		
	November-87	75,000		
DSM IV	May-94	Not given	May 1986 - January 1992	1995.6
	July-94	Not given		
	August-94	Not given		
	January-95	Not given		

market share, and therefore, the introduction of MeruvaxII® and MMRII® would be the first fetal cell vaccines to impact the US, and correspond to the 1980 to 1981 autistic disorder change point. The US 1988.4 change point corresponded to the addition of a second dose of MMRII® to a measles vaccination campaign that increased compliance from ≤50 to 82% between birth years 1987 and 1989 (Centers for Disease Control 1989; Kaye and Jick 2001) as well as to the introduction of Poliovax in 1987. The 1995.6 autistic disorder change point corresponded to the approval and introduction of the Varicella vaccine (Varivax®). The Western Australia 1990 autistic disorder change point came shortly after the 1989 addition of MMR vaccine to the vaccination schedule, supplied solely with MMRII® (Table 3).

Association between autistic disorder and fetal cell manufactured vaccination coverage

US autistic disorder prevalence began rising after birth year 1978 (Newschaffer and Gurney 2005), and has continued to rise through birth year 2008. Figure 3A illustrates the continuing rise in US autistic disorder for 8 year olds born between 1992 and 2003. IDEA data for 3 year olds (not shown) through birth year 2008 demonstrates a continuing rise in US autistic disorder after 2003. Figure 3B illustrates that varicella coverage increased steadily after its approval in 1995 for children whose birth years were 1993 through 1998 to 1999, leveling off after reaching just over 80% saturation.

Hepatitis Avaccine (Havrix®) was approved for use in the US in 1995; however, it was neither part of the childhood immunization schedule nor recommended for use by any states. In 1999, 17 states began recommending/ considering its use for children 24 months and older, and in 2005 it was included in the ACIP recommended vaccination schedule for children 12 months and older (Table 3). Hepatitis A coverage (Figure 3D) shows a more complicated compliance due to the non-uniform state recommendations from 1999 through 2005. Based on approval dates and recommendation dates, Hepatitis A use could have affected autistic disorder rates for children born in 1997 or later, however, there is not public data tracking vaccination rates prior to 2006. Extrapolating from age of immunization to birth years, Hepatitis A immunization coverage has increased steadily for birth year 2003 through birth year 2008 (Figure 3D).

To compare absolute numbers of children diagnosed with autistic disorder to the absolute numbers of children vaccinated with Varivax®, we performed linear regression analysis for birth years 1992 to 1998, during which time Varivax® coverage increased linearly. Additionally, birth years 1992 to 1998 were chosen because state variation in use of Hepatitis A vaccine after 1999 confounds the use of Varivax® as a measure of exposure to vaccines manufactured in human fetal cell lines for birth years subsequent to 1998. Figure 3C illustrates the highly significant correlation between the absolute number of children vaccinated with Varivax® and the absolute number of children diagnosed with autistic disorder (R^2=0.8774; P<0.001). A similar strong correlation was also observed between the number of children vaccinated against Hepatitis A and the number of autistic disorder cases for birth years 2003 to 2008 (R^2=0.6762; P<0.001).

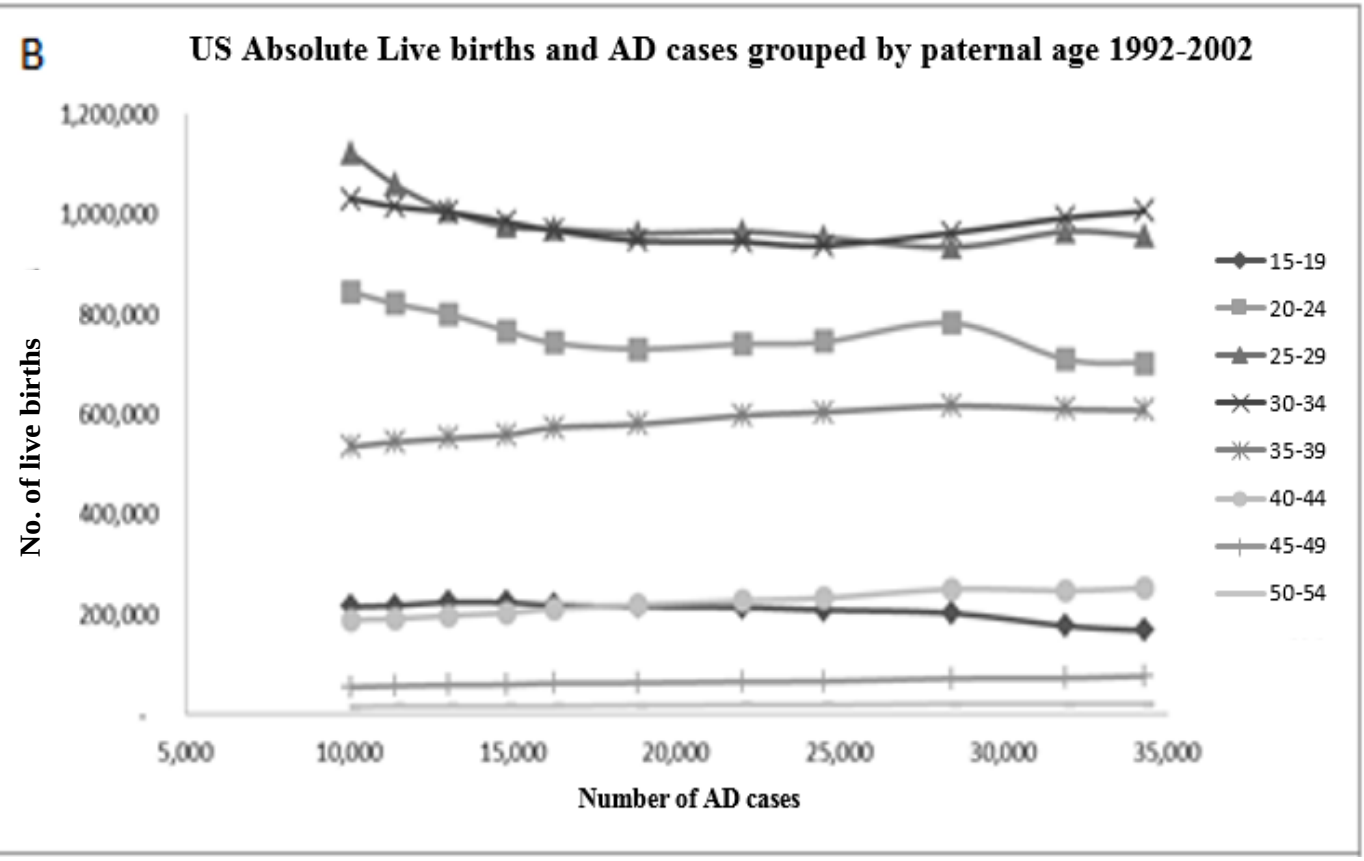
DNA residuals in human fetal cell line manufactured vaccines

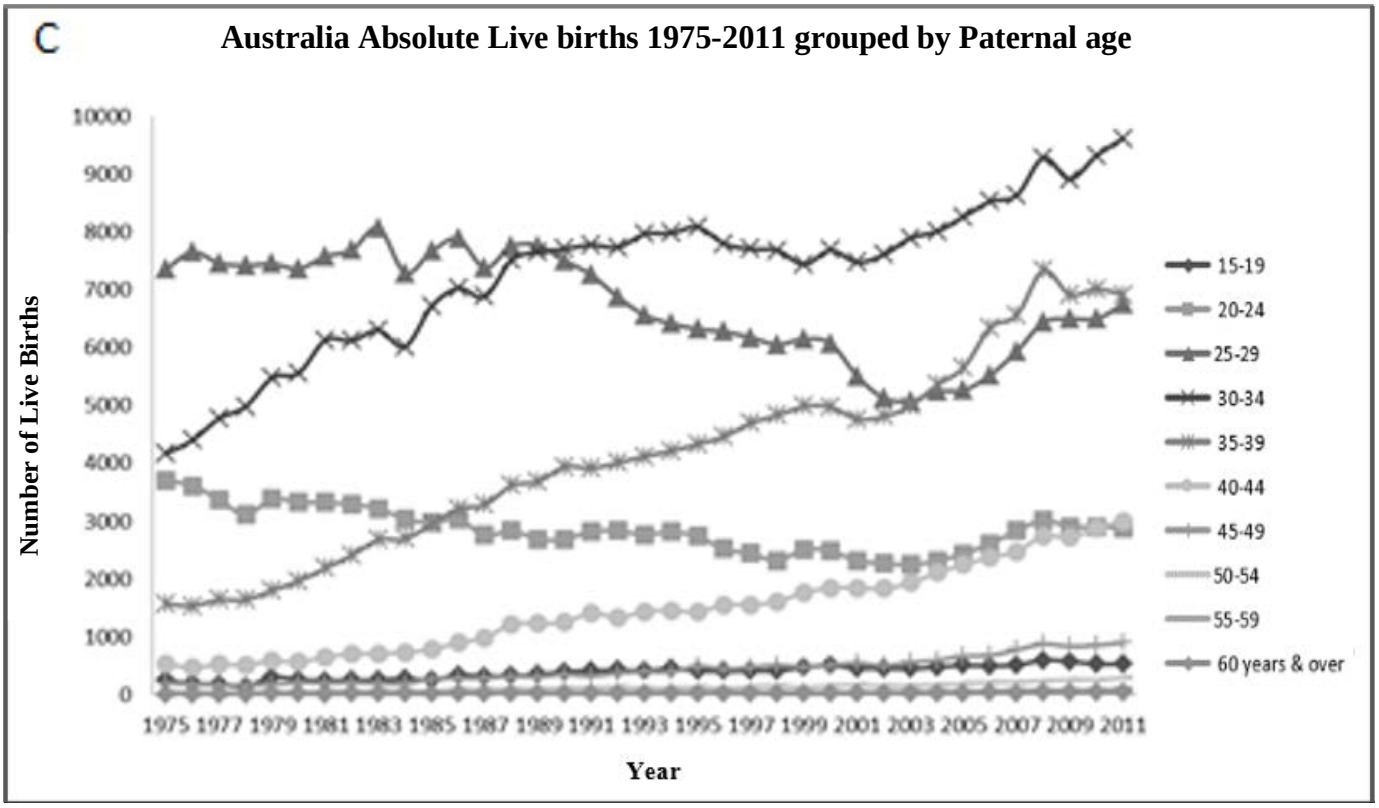
In addition to the ingredients listed on the package

A


US Absolute live births from 1960-2008 grouped by paternal age
Number of live births
1,400,000
1,200,000
1,000,000
800,000
600,000
400,000
200,000
-
1960
1965
1970
1975
1980
1985
1990
1995
2000
2005
2010
2015
Year
15-19
20-24
25-29
30-34
35-39
40-44
45-49
50-54

B


US Absolute Live births and AD cases grouped by paternal age 1992-2002
No. of live births
1,200,000
1,000,000
800,000
600,000
400,000
200,000
-
5,000
10,000
15,000
20,000
25,000
30,000
35,000
Number of AD cases
15-19
20-24
25-29
30-34
35-39
40-44
45-49
50-54



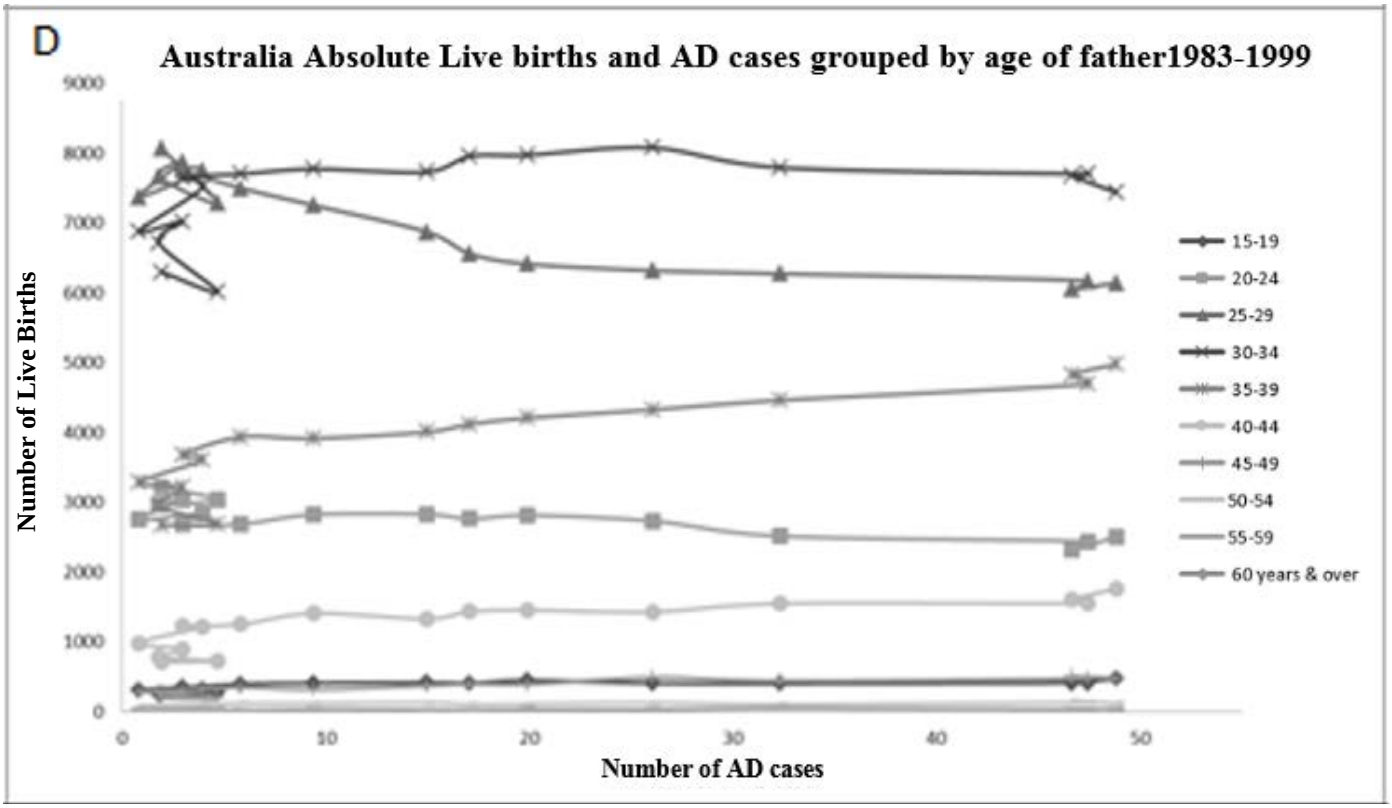


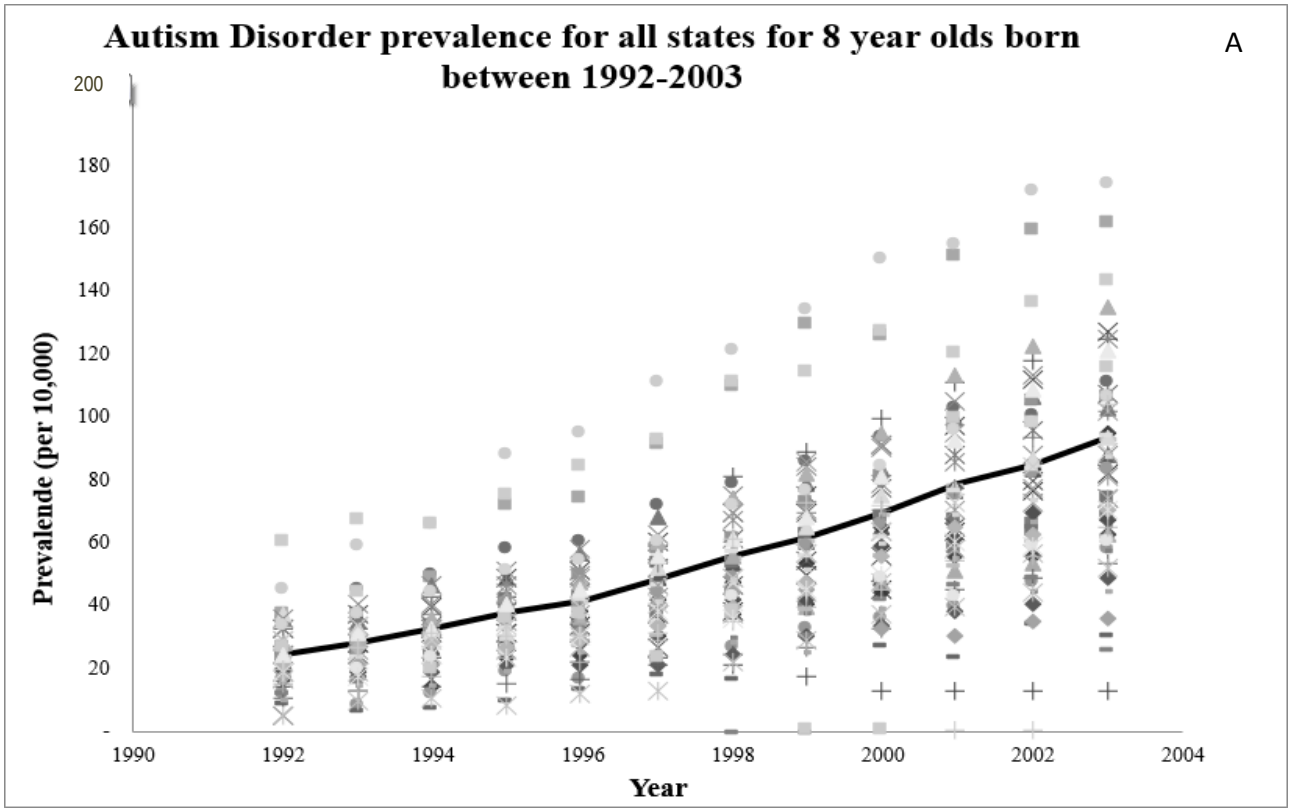
Figure 2. Number of live births grouped by paternal age and its correlation with AD cases over years of US and Western Australia. Panel A and C show number of live births at different father's age from 1960 to 2008 for the U.S. and from 1975 to 2011 for Western Australia. Panel B and D show the number of live births and number of AD cases by paternal age of the U.S. and Western Australia.

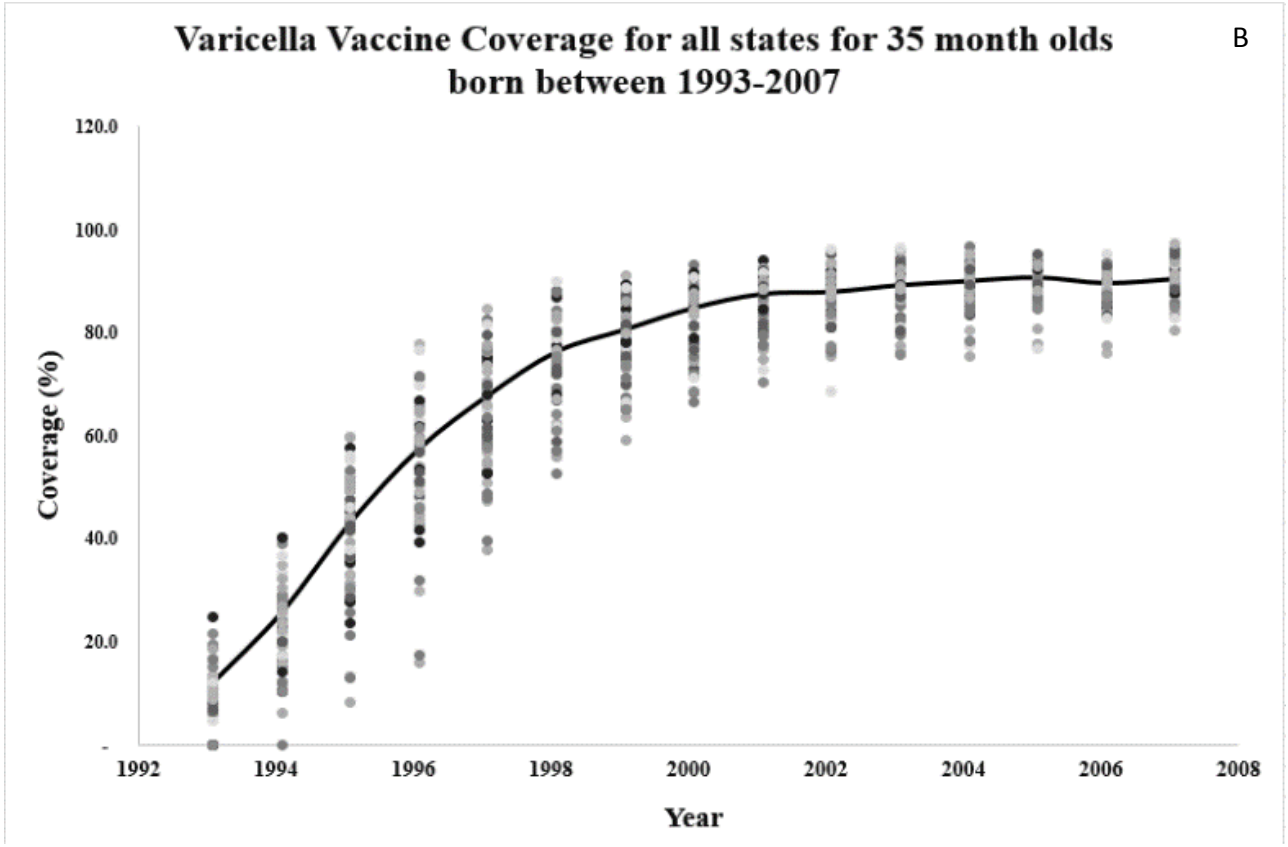
Table 3. History of MMR, varicella and hepatitis A vaccines approved for use in the US, UK and Western Australia manufactured using human fetal cell lines and contaminated with human fetal DNA and/or retroviral fragments.

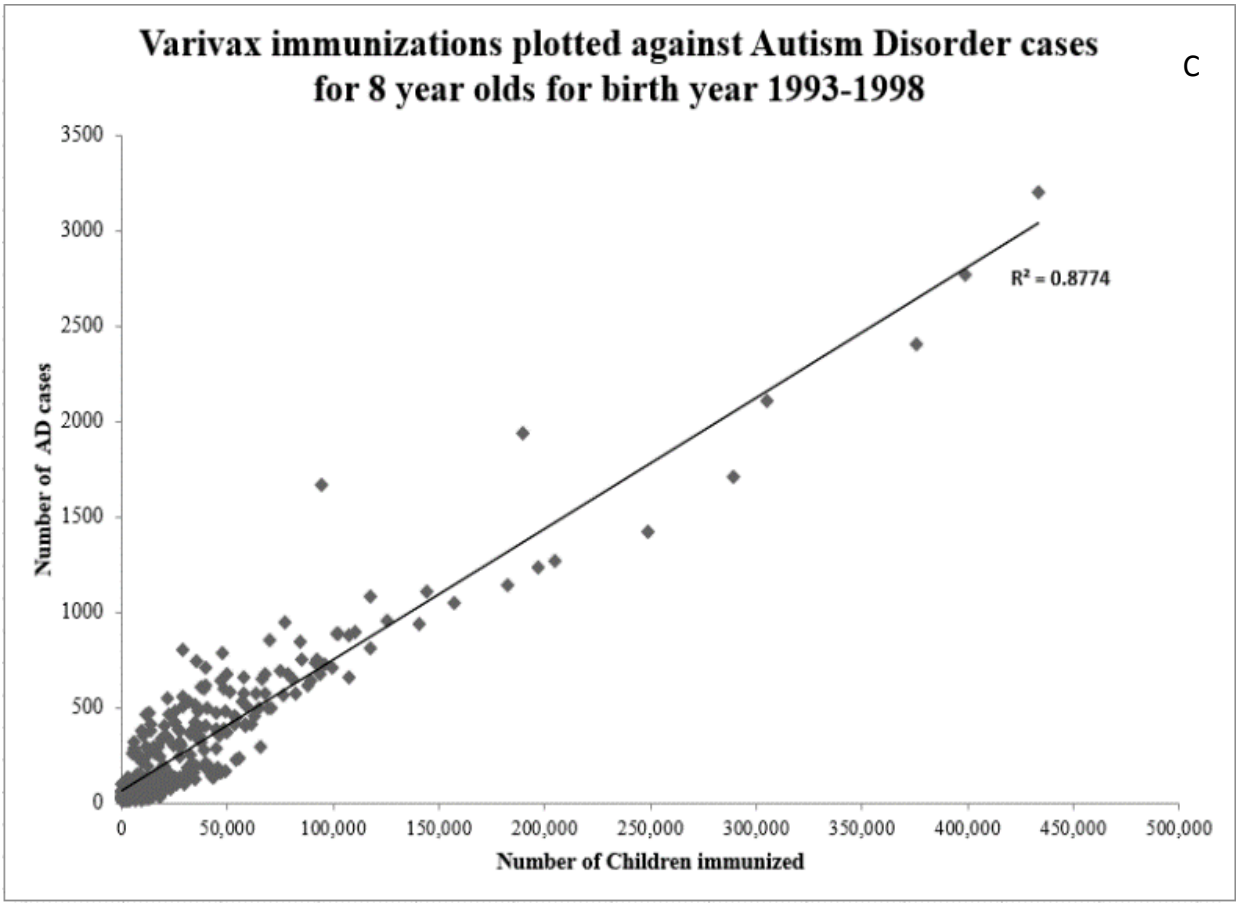
Date	Vaccine name	Type of vaccine	Manufacturer	Age of immunization	Events
			History of vaccines approved for use in US		
01/1979	Meruvax II	A rubella vaccine with the RA 27/3 (human diploid fibroblast)	Merck	>=12 months	Licensed.
1979	MMR II	Combined measles, mumps and rubella with the RA 27/3 strain	Merck	>=12 months	Licensed
3/17/1995	Varivax	Varicella virus vaccine, live	Merck	>=12 months	Licensed
2/22/1995	Havrix	The first inactivated hepatitis A vaccine	SmithKline Beecham	>=24 months	Licensed
3/29/1996	Vaqta	A second inactivated hepatitis A vaccine	Merck	>=24 months	Licensed
1999	Vaqta				17 states considered for use
5/11/2001	Twinrix	A combined hepatitis A inactivated and hepatitis B (recombinant) vaccine	SmithKline Beecham	>=18 years	Licensed
8/11/2005	Vaqta	A second inactivated hepatitis A vaccine	Merck	>=12 months	FDA approved lowering the age limit to 12 months
2005	Vaqta				Included in ACIP recommendations
10/18/2005	Havrix	The inactivated hepatitis A vaccine	GSK	>=12 months	FDA approved lowering the age limit to 12 months
3/28/2007	Twinrix	A combined hepatitis A inactivated and hepatitis B (recombinant) vaccine	GSK	>=18 years	FDA approved an accelerated dosing schedule
			History of vaccines approved for use in Australia		
1989	MMR II	Combined measles, mumps and rubella with the RA 27/3 strain	Merck, Sharp, Dohme - MSD	>=12 months	Licensed
1999	Varivax	Varicella virus vaccine, live	Merck, Sharp, Dohme - MSD	>=12 months	Licensed
1999	Varilrix	Varicella virus vaccine, live	GSK	>=12 months	Licensed
			History of vaccines approved for use in UK		
10/1988	MMR II	Combined measles, mumps and rubella with the RA 27/3 strain	SmithKline Beecham, Merieux, Merck Sharpe	>=12 months	Licensed
			History of vaccines approved for use in Denmark		
1987	MMRII	Combined measles, mumps and rubella with the RA 27/3 strain	Statens Serum Institut	15 months and 12 years of age	Licensed
1989	MMRII	Combined measles, mumps and rubella with the RA 27/3 strain	Statens Serum Institut	< 18 years	Extended

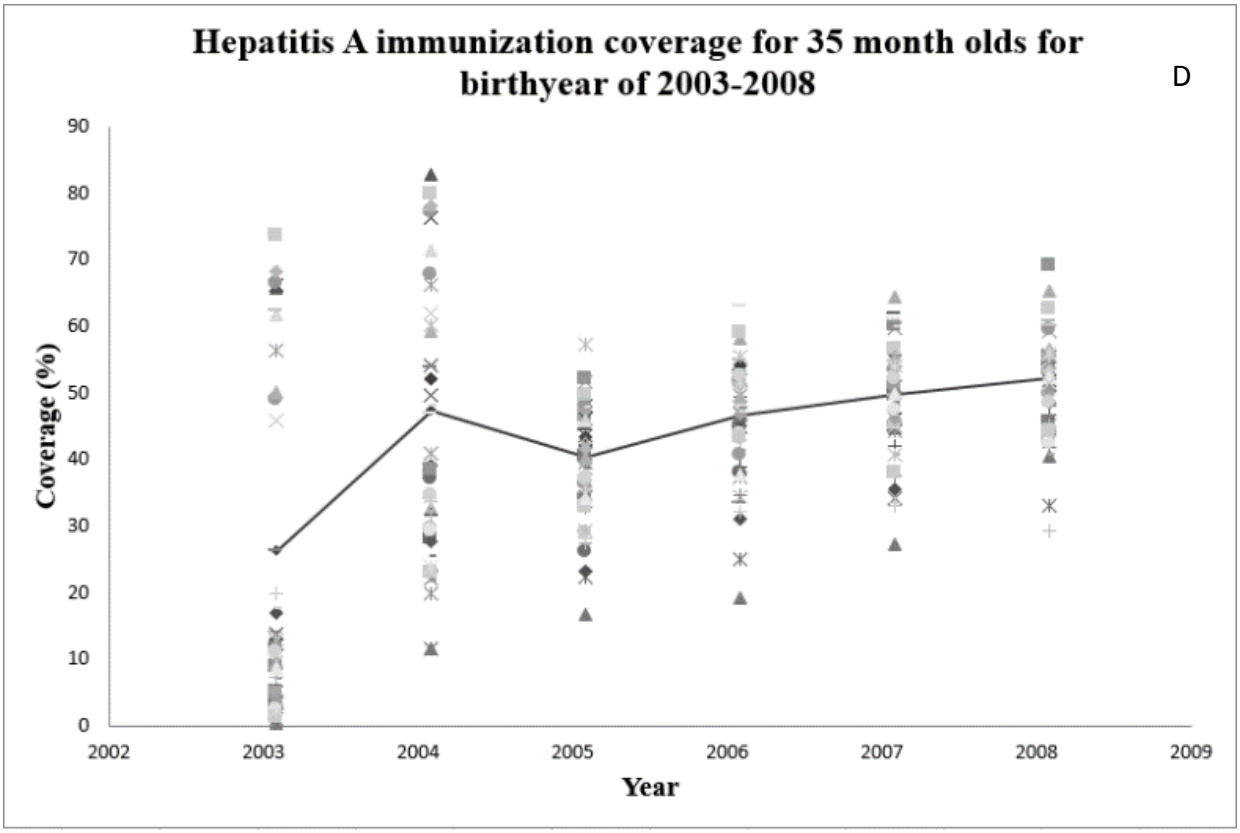
insert for Meruvax II® (rubella), we detected significant levels of human ssDNA (142 ± 8 ng/vial) as well as dsDNA (35 ±10 ng/vial) fragmented to ~215 base pairs in length. The MMR II® package insert discloses the presence of human fetal residuals nor how much cell substrate dsDNA or ssDNA contaminates each dose. In each vial of Havrix® , we detected ssDNA (301 ± 153 ng/vial) as well as dsDNA (44 ± 24 ng/vial) unfragmented residual DNA more than 48.5 K base pairs in length. The Havrix® package insert discloses the presence of human fetal cellular residuals from the MRC-5 cell line, but not the DNA contaminant levels specifically.

The Varivax® vaccine is manufactured using the human diploid cell line MRC5, and is contaminated with 2 micrograms of cell substrate double stranded DNA. Single stranded DNA levels are not reported in Merck's Varivax Summary Basis for Approval document nor are the length of the DNA fragments contaminating the vaccine (Merck 2011).


Autism Disorder prevalence for all states for 8 year olds born between 1992-2003
A
Prevalende (per 10,000)
200
180
160
140
120
100
80
60
40
20
-
1990
1992
1994
1996
1998
2000
2002
2004
Year


Varicella Vaccine Coverage for all states for 35 month olds born between 1993-2007
B
Coverage (%)
120.0
100.0
80.0
60.0
40.0
20.0
-
1992
1994
1996
1998
2000
2002
2004
2006
2008
Year


Varivax immunizations plotted against Autism Disorder cases for 8 year olds for birth year 1993-1998
C
Number of AD cases
Number of Children immunized
R² = 0.8774
0
500
1000
1500
2000
2500
3000
3500
0
50,000
100,000
150,000
200,000
250,000
300,000
350,000
400,000
450,000
500,000


Hepatitis A immunization coverage for 35 month olds for birthyear of 2003-2008
D
Coverage (%)
Year
0
10
20
30
40
50
60
70
80
90
2002
2003
2004
2005
2006
2007
2008
2009

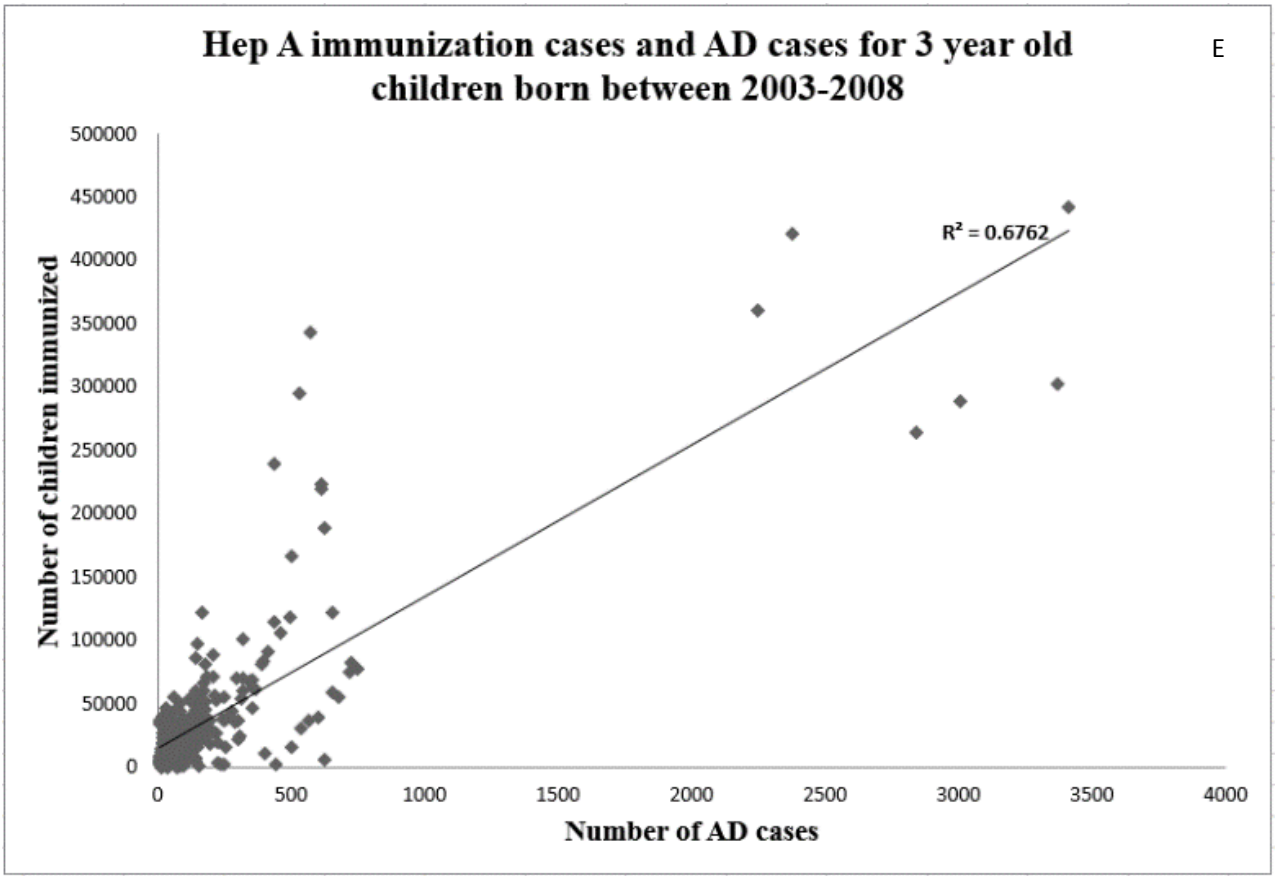


Figure 3. Varicella® and Hepatitis A vaccine coverage trend and their correlations with AD cases over years in the U.S. Panel A shows the increase in AD prevalence for children born between 1992 and 2003. Panel B and D show Varivax and Hepatitis A coverage for all states. Panel C and E show the high correlation between the numbers of children vaccinated with Varivax® and Hepatitis A with AD cases.

DISCUSSION

Autistic disorder begantorise in the US after birth year 1978 (Newschaffer and Gurney 2005). According to EPA recommendations, birth year change points for prevalence of autistic disorder should drive consideration of environmental triggers, as for any disease (McDonald 2010). In this study, we report three calculated US autistic disorder birth year change points for birth years 1970 through 2002. Iterative fitting algorithms identified 1980.8 (1980.4 to 1981.2), 1988.4 (1987.8 to 1989) and 1996.5 (1994.6 to 1996.6) as 'change point' years for US autistic disorder prevalence. While no reporting system is perfect, we have tried to minimize any effects of erroneous diagnosis or coding by choosing the narrower autistic disorder or infantile autism. Regardless of the cause(s) diagnoses of autistic disorder have risen dramatically, adding a significant public health burden and therefore demanding critical assessment of environmental triggers that may be responsible for this apparent epidemic. Candidate environmental triggers should have the following attributes: exposure from conception to at least 3 years of age around each change point, absent or substantially lower prior to the first identified change point, a dose-effect associated with calculated change points, and toxicological mechanisms compatible with disruption in early neural development, that is, biological plausibility.

Therefore, we asked the question whether information about diagnostic criteria would predict autistic disorder change points consistent with our calculated autistic disorder change points. Even though changes in diagnostic criteria have clearly occurred, examination of DSM revisions suggests that autistic disorder (not the broader ASD) diagnosis has not been relaxed. DSM IV introduced a requirement to exclude Rett's disorder, implying that DSM-IV may be more restrictive than DSM-III or IIIR. Interestingly, the DSM manual is not typically listed among the diagnostic tools used by any of the practitioners when making their initial diagnosis of either autistic disorder or ASD anyway (Wiggins et al., 2006). More importantly, we analyzed only autistic disorder data; excluding datasets that contained ASD diagnoses, consistent with the CDC statement that a child with autistic

disorder “can be less complicated to diagnose than other spectrum disorders” (Victoria et al., 2010).
Regardless of whether autistic disorder diagnostic relaxaion has or has not occurred, and regardless of whether

DSM is used as a tool for initial autistic disorder diagnosis or not, predicted autistic disorder birth year change points based on DSM revision printing schedules do not correlate with calculated autistic disorder change points and cannot be the primary environmental or sociological trigger responsible for current autistic disorder prevalence.

Multiple publications over the past several years point to the potential importance of protein disrupting de novo point mutations in the etiology of autism spectrum disorders and other childhood onset ND diseases. In the US, advancing paternal age has an apparent association with these disorders, if one looks only at dates from 1980 onward. However, as shown here, consideration of live births to older fathers back to 1960 disputes the importance of paternal age as a primary trigger for the increased prevalence of autistic disorder. Autistic disorder diagnosis was low and stable from birth year 1960 through 1978. Furthermore, in their publication on advanced paternal age and de novo mutations by Kong et al., 2012 point out that live births to older fathers in ice land were substantially higher from 1650 through 1940 than they are today (Kong et al., 2012), time periods when autistic disorder was extremely rare. Additionally, no studies have been done to determine if the de novo mutations in children with ND disease are occurring in the spermofolder fathers or in the somatic cells of the children. However, paternal age has been found to be a risk factor for autism spectrum disorder diagnosis (Kong et al., 2012). Our data, taken together with the evidence that advancing age is associated with sperm susceptible to double-strand break formation and genomic instability (McDonald 2010), may explain the association between paternal age and childhood ND.

In 1979, coincident with the first autism disorder change point, vaccine manufacturing changes introduced human fetal DNA fragments and retroviral contaminants into childhood vaccines (Victoria et al., 2010). While we do not know the causal mechanism behind these new vaccine contaminants and autistic disorder, human fetal DNA fragments are inducers of autoimmune reactions, while both DNA fragments and retroviruses are known to potentiate genomic insertions and mutations (Yolken et al., 2000; Kurth 1998; U S Food and Drug Administration 2011). Infants and children are almost universally exposed to these additional vaccine components/contaminants, and these converging events are associated with rising autistic disorder in a dose-dependent fashion due to the increasing numbers of human fetal manufactured vaccines which have been added to the US immunization guidelines, including Pentacel®, which since 2008, contains inactivated polioviruses grown on the MRC-5 human fetal cell line. Pentacel® is recommended for children at 2, 4 and 6 months of age, and may account for the recent idea that scientists have become more adept at diagnosing autism at younger age. Diagnosis at younger age may more likely be the result of introducing human fetal cell vaccine contaminates to younger children.

Vaccines that have been cultured on or manufactured using the WI-38 fetal cell line such as MeruvaxII®, MMRII®, Varivax®, Havrix® and Pentacel® are additionally contaminated with fragments of human endogenous retrovirus HERVK (Victoria et al., 2010). Recent evidence has shown that human endogenous retroviral transcripts are elevated in the brains of patients with schizophrenia or bipolar disorder (Frank et al., 2005), in peripheral blood mononuclear leucocytes of patients with autism spectrum (Freimanis et al., 2010) as well as associated with several autoimmune diseases (Tai et al., 2008). The strong ecological association between human fetal cell line-manufactured vaccines and autistic disorder change points calls for further investigation of these childhood vaccine contaminants, and for the sake of preserving critical vaccination coverage, even a return to animal-based manufacturing.

Manufacture of childhood vaccines in human fetal cell lines, with its associated retroviral and human DNA fragment contaminants, fulfills all of the necessary requirements as a primary trigger for the ND disease, autistic disorder. The contaminants were not present prior to the first US autistic disorder change point, they have continued to increase the environment with additional human fetal vaccine approvals and doses, and they have clinically documented adverse immunologic and mutagenic side effects. With the 2008 US approval of Pentacel® for children at 2, 4, and 6 months of age, we may be seeing age of onset of regressive autism decrease dramatically.

This study is the first laboratory and ecological study conducted to date that has examined the question of a relationship between human fetal cell line manufactured vaccines and autism. Autistic disorder diagnosis has typically not been made until the age of 5, and confirmed diagnosis is often not made until the age of 8 (Lord et al., 2006; Luyster et al., 2009) .Therefore, we were not able to investigate direct correlations between autistic disorder prevalence and vaccine coverage of other human fetal cell manufactured vaccines approved after Hepatitis A, such as the Pentacel® vaccine. Nevertheless, between birth year 1992 through birth year 1998, there are sufficient numbers of children vaccinated or not vaccinated with Varivax® (chickenpox), whose data is maintained in the Vaccine Safety Datalink (VSD), that could be used to determine the relative risk of an autistic disorder diagnosis for those who did or did not receive this heavily contaminated fetal cell manufactured vaccine (Yolken et al., 2000). This overlooked potential trigger for the worldwide autism disorder epidemic demands additional studies in order to assure the safe manufacture of routine recommended childhood vaccines, particularly since reverting to animal based manufacturing methods

is readily available.

ACKNOWLEDGMENTS

This work was supported by a grant from the Murdock Charity Trust and our donors.

Ethics statement

All data used in this manuscript were from public data files and therefore is exempted from IRB approval according to guidelines from The National Human Subjects Protection Advisory Committee (NHRPAC) recommendations on Public Use Data Files approved at the January 28 to 29, 2002 Committee meeting. (http://www.hhs.gov/ohrp/archive/nhrpac/documents/datal tr.pdf): “Responsibility of Users of Public Use Data Files: Users of public use data files do not need to obtain IRB approval to use such files or seek a determination that the use of the public data files meets the criteria for being exempt from IRB review.”

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Factors associated with endemicity of *Yersinia pestis* in Namwala District of Zambia

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Plague which is a flea borne zoonotic disease of mammals caused by the bacterium Yersinia pestis has occurred in Eastern and Southern parts of Zambia as epizootics. This study was conducted to determine factors associated with these outbreaks. The study was done in Namwala district of Zambia and a cross-sectional study design was used. The two stage cluster sampling technique was used. The first stage involved conveniently identifying the 8 villages where human cases of plague had been reported. The second stage was random selection of households within the villages. These were sampled without prior knowledge of whether the household had a case of human plague or not. The sampling unit was the households. A total of 45 households were sampled. Twenty six (42%) of the households reported to have had a human case. The mean age of these cases was 10.86 ± 6.74 years while 74% of these were males. The households who reported cases and those who did not report cases were not different in bush activities they were involved in, type of housing they lived in and in terms of floors of their respective houses. The households reporting cases as compared to those who did not report cases were more likely to have rodents with plague found in their surrounding (94.7% vs 73.1%), have dirty surroundings (84.2% vs 50%), have a radius of ≤ 20 meters as nearest human dwelling (94.7% vs 53.8%) and have unplastered walls of their houses (84.2% vs 38.5%) (P < 0.05). The entry of infected rodents with fleas to the human habitat and the contact of fleas with humans contribute to the outbreak of plague under conditions which favour survivor of fleas like unplastered houses, dirty surroundings and the existence of infected rodents within a household surrounding of 20 m or less. Employing measures which minimizes the contact between fleas and humans can reduce outbreaks.

Key words: Endemicity, *Yersinia pestis*, Namwala District, Zambia.

INTRODUCTION

Plague is a flea-borne zoonotic disease of mammals caused by the bacterium *Yersinia pestis* which has an incubation period of 2 to 6 days in humans. Rodents are primarily infected by plague and act as reservoirs (Butler, 2000; Perry and Fetherston, 1997). Certain species of rats, prairie dogs, vole, mouse, squirrels, dogs, cats, and rabbits are also suspected to be reservoirs (Guiyole et al., 1994; Perry and Fetherston, 1997). Generally, *Y.*

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pestis is maintained in populations of wild rodents and their fleas as an obligate parasite, which has been recovered from over 200 mammalian species worldwide (Gasper and Watson, 2001). There are three principal clinical presentations of plague namely bubonic, septicaemic and pneumonic plague. It has a very high fatality rate of 50 to 60% if left untreated. At the moment, plague circulates in rodent populations on five continents except Australia and Antarctica (Butler, 2000). Most human cases of plague are reported from developing countries in Asia and Africa. In Africa, plague foci are distributed throughout the continent (Mwase et al., 1999).

Transmission may occur in several ways with the most efficient involving the ingestion of the organism by the flea during a blood meal from a bacteremic host (Bacot and Martin, 1914). Transmission of *Y. pestis* from fleas to humans occurs primarily via the bites of infected fleas (Perry and Fetherston, 1997). Human to human transmission occurs in pneumonic plague which is highly infectious and rapidly fatal (Wren, 2003). This is where the spread occurs via the respiratory droplets between humans; however this type of epidemic is currently uncommon due to the advent of effective antibiotics and modern public health measures. The other mode of transmission is by inhaling droplets expelled by the coughing of a plague-infected animal (Doll et al., 1994). This can result in plague of the lungs (plague pneumonia).

In Zambia, plague has been reported in Lusaka, Eastern, Western, Northwestern and Southern regions (Mwase et al., 1999) and currently it has occurred in the Eastern and Southern parts of the country as periodic epizootics (Hang'ombe et al., 2012).The first confirmed major outbreak of plague in Zambia was in the Southern region of Namwala district in December, 1996 to February, 1997, where 267 human cases were reported, and 26 people died (Hang'ombe et al., 2012). Following this major outbreak, cases are being reported on an annual basis. This study was conducted in Kabulamwanda area of Namwala district where isolated cases were reported in 2012 and rats were confirmed as harbouring plague. This is according to information obtained from the Namwala District health office in Zambia. The study was undertaken to determine the factors associated with plague in this area.

MATERIALS AND METHODS

Study area

The study was conducted in Kabulamwanda area of Namwala district in the Southern province of Zambia where outbreaks are reported annually. Eight villages of Kabulamwanda where the disease usually occurs annually were included in the study. The villages sampled included Nacubi, Nalubwe, Njiri, Shamani, Shimalambwe, Shimuhila, Shimusashi and Shitongo. Some ongoing studies in the area have confirmed the presence of *Y. pestis* in these areas sampled (Hang'ombe et al., 2012).

Sampling

A cross-sectional study design was employed and the two stage cluster sampling technique was used. The first stage involved conveniently identifying the villages where human cases of plague had been reported. The second stage was random selection of households within the villages. These were sampled without prior knowledge of whether the household had a case of human plague or not. The sampling unit was the households. The sampled houses were 45. From these households 19 were reported to have had cases while 26 did not have any cases. The non cases acted as controls for this study.

Survey

The questionnaire was administered by interviewing household members in different households. Apart from enquiring whether there was a plaque case(s) reported on a particular household, the survey also addressed environmental variables such as hut specifications, movements of rodents in or around the huts after farm harvesting and during the rainy season, and basic human behaviors. Hut specifications included; type of houses (mud or brick and plastered, mud or brick and not plastered) and type of floor (mud and temporal, concrete and permanent). Human behavior questions included activities done by the household members either in the bush or plains (such as cattle herding, collection of firewood and poles or activities that allow an individual to venture out in the bush or forest). Moreover, plague control/prevention measures practiced by different households and when these measures are implemented was also noted. Knowledge on the treatment of plague was also assessed for different households. Age and sex variables were only recorded for the plague cases. Observations were also made on the cleanliness of the households and their respective surroundings. Clean surrounding was classified as having a radius of 20 m or more within the house which was clean and cleared, while a clean radius of 20 m or less were classified as having dirty surrounding. Coordinates (latitude and longitude) together with the altitude of each particular household were measured by global positioning system (GPS) reader and recorded.

RESULTS

A total of 45 households were sampled from the 8 villages in Kabulamwanda area of Namwala district. From Table 1, the reported numbers of human cases were 19, thus 42.2% of the sample. The mean age of the cases was 10.86 ± 6.74 years. Of the households sampled 16 (35.6%) had a clean surrounding while 29 (64.4%) had a dirty surrounding. Twenty nine households (64.4%) were involved in cattle herding and collection of firewood while 9 (20%) were involved in other activities in the bush/plains. Seventy four percent of the cases were males while 26% were females (Figure 1). Table 2 shows that thirty seven (82.2%) of the households with plague had rodents found within their surroundings. Sixty eight percent of households reported that rodents increase after harvesting and 62.2% reported flea increase during the rainy season. Seventy one percent of the households had a human dwelling of less than or equal to 20 meters from where rodents with plague were found. On the other hand fleas were reported to be on the increase during

Table 1. Human attributes of the sampling information.

Variable	Category	n (%)
Reported human cases of plague, N=45	Yes	19 (42.2)
	No	26 (57.8)
Age of plague cases	Mean (SD)	10.86 (6.7)
Surrounding area of home, N=45	Clean	16 (35.6)
	Dirty	29 (64.4)
Bush/plain activities cases were involved in, N=76	Cattle herding and collection of firewood	58 (76.3)
	Other activities	18 (23.7)

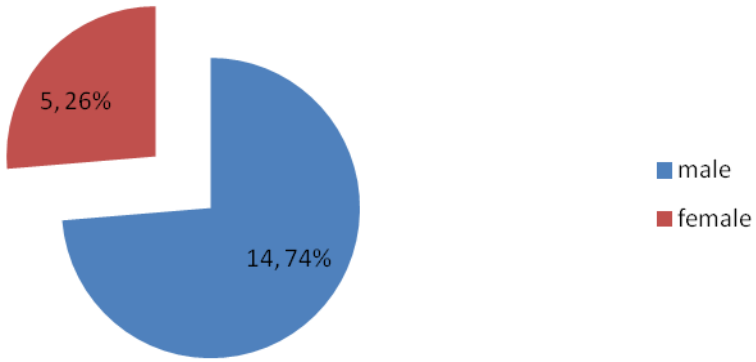

Figure 1. Sex of plague cases number

Table 2. Rodent attributes of the sampling information.

Variable	Category	n (%)
Nearest human dwelling where rodent was found	> 20 m	10 (22.2)
	≤ 20 m	32 (71.1)
Rodents with plague found in the surrounding, N=45	Yes	37 (82.2)
	No	68 (17.8)
Rodents specie which tested positive for plaue, N=45	*Gerbillurus*	41(91.1)
	Other (*rattus/mastomys*)	4 (8.9)
Reported time of year when rodent increase, N=41	Rainy season	10 (22.2)
	After harvesting	31 (68.9)
Reported time of year when rodent decrease, N=39	Cold season	12 (26.7)
	Rainy season	27 (60.0)

rainy season while they are reported to reduce during cold season (Table 3). Table 4 shows that eighty two percent of the housing units were made of mud and more than half of these units were not plastered (57.8%). 80%

Table 3. Flea attributes of the sampling information.

Variable	Category	n (%)
Reported time of year when fleas increase, N=41	After harvesting	13 (28.9)
	Rainy season	28 (62.2)
Reported time of year when fleas decrease, N=39	Cold season	29 (64.4)
	Rainy season	10 (22.2)

Table 4. Characteristics of housing units in the households sampled.

Variable	Category	n (%)
Type of housing, N=45	Brick	8 (17.8)
	Mud	37 (82.2)
Type of wall, N=45	Plastered	19 (42.2)
	Not plastered	26 (57.8)
Type of floor, N=45	Concrete	9 (20.0)
	Temporal	36 (80.0)

of these units had temporal (not concrete) floor as compared to 20% which had permanent (concrete) floor. Almost all these households grew corn near their homes.

Households reporting cases compared with those who did not report cases exhibited no significant differences in activities such as cattle herding, firewood collection and other bush activities, P > 0.05 (Table 5). Households reporting plague cases were significantly more likely to have had rodents with plague found in their surrounding (94.7% vs 73.1%, P=0.014). Those households with a dirty surrounding (84.2% vs 50%, P = 0.018), with a radius of greater than or equal to 20 m as nearest human dwelling (94.7% vs 53.8%, P = 0.013) and those with walls which were not plastered (84.2% vs 38.5%, P = 0.002) were also significantly more likely to report human plague cases. According to information obtained from the medical offices in Namwala district, the disease is on the increase from February, March up to May. And when there are suspected cases, bubos are checked for human cases by taking specimens from the swelling. Confirmed cases are isolated and the maximum number of days of admission is 7 days with Doxycyline given once every 7 days for prophylaxis, with chloramphenicol and gentamicin being used for treatment.

DISCUSSION

Factors associated with plague in endemic areas of the disease in Zambia have not been studied extensively. In this study, 42.3% of the households had reported b a human plague case. The mean age of the cases was 10.9 years showing that the disease affect mostly young children. This is supported by a study done in Zimbabwe which indicated that plague was associated with an age of 10 years or older (Manungo et al., 1998). The larger proportion affected by this disease are male children probably because most male children are more adventurous and may be linked to the hunting, killing and touching of the rodents and may be involved in many other outdoor activities. More than half of these households had dirty surroundings while most of them were made of mud and not plastered. Interestingly, member's involvement in bush activities, type of housing and floor were not associated with plague cases. This is in line with a study done in New Mexico which indicated that plague was as a result of entry of the reservoir host into the habitat of human rather than from entry of human into the sylvatic habitat of the reservoir host (Jonathan et al., 1979). Coming to control and treatment measures known by people, most of them reported using cats for control of rodents and taking patients to health center for treatment.

Human plague cases were associated with rodents with plague found in the surrounding area, and within a human dwelling of less than or equal to 20 m. A dirty surrounding area within the household and unplastered houses were also associated with plague cases. This could be as a result of rodents hiding in the dirty surrounding and themselves being infected with plague. The wall which is not plastered could also be the hiding area for fleas which can easily infect the humans . Eventhough

Table 5. Risk factors associated with reported cases of human plague.

Variable	Household reported a case (n=19)	Household did not report a case (n=26)	P -value
Rodents with plague found in surrounding area	18 (94.7)	19 (73.1)	0.014
Surrounding area of home dirty	16 (84.2)	13 (50.0)	0.018
Cattle herding and collection of firewood as bush activities cases were involved in	12 (63.1)	17 (65.4)	0.703
≤ 20 m as nearest human dwelling were rodents were found	18 (94.7)	14 (53.8)	0.013
Type of housing being mud	17 (89.5)	20 (76.9)	0.435
Type of wall, not plastered	16 (84.2)	10 (38.5)	0.002
Type of floor, temporal	16 (84.2)	20 (76.9)	0.712

the rodents were reported to increase in the surrounding areas of households after harvesting, the fleas were interestingly reported to increase in the rainy season. It is also during the rainy season that cases are reported to be on the increase. The reason could be that during the rainy season rodent barrels are flooded, resulting to rodents dying. The fleas which were on these rodents go to humans as the next hosts especially in the plains, to cause plague. This is in contrast to the study done in Vietnam where the incidence of plague is at its peak during the dry season (Pharm et al., 2009). This Vietnam study further reported that the risk of plague occurrence was associated with an increased monthly flea index and increased rodent density (Pharm et al., 2009). This agrees with the finding of our current study which indicates that when there is flea increase there is also plague increase in the rain season.

Maize (corn) was grown nearly in almost each and every household sampled. According to a study done in West Nile region of Uganda, they were able to identify potential residence-based risk factors for plague associated with huts within historic case or control villages (for example, distance to neighboring homestead and presence of pigs near the home) and huts within areas previously predicted as elevated risk or low risk (for example, corn and other annual crops grown near the home, water storage in the home, and processed commercial foods stored in the home) (MacMillan et al., 2010). To effectively control the transmission of plague we suggest that disease control measures which have been suggested be employed in these endemic areas. These control methods which have been suggested and used in disease management include preventive measures such as rat proofing buildings and also use of insecticide and repellents. Control of contact with patients and their immediate environment has also been suggested and used.

CONCLUSION

The entry of infected rodents with fleas to the human habitat and the contact of fleas with humans contribute to the outbreak of plague especially under conditions which favour the survivor of fleas like unplastered houses, dirty surroundings and the existence of infected rodents within a household surrounding of 20 m or less.

RECOMMENDATIONS

Based on the results the following recommendations are made.

1. To effectively control the transmission of plague it is suggested that disease control measures such as use of insecticide to eliminate the fleas should be implemented in endemic areas.
2. People should be encouraged to plaster the houses whether brick or mud. They should also be encouraged to keep surrounding areas of the homes clean for a radius of 20 or more meters to keep away the bush rats.

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