Molecular epidemiology of a household outbreak of Shiga-toxin-producing *Escherichia coli* in Poland due to secondary transmission of STEC O104:H4 from Germany

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We characterized two STEC O104:H4 clinical isolates collected in Poland from a 7-year-old boy with haemolytic uraemic syndrome (HUS) and his nanny. This household outbreak began on 29 May 2011. Because of its time-frame, the outbreak was assumed to be part of the international STEC O104:H4 outbreak that arose in Germany in May 2011. The two Polish isolates were Shiga-toxin-producing *Escherichia coli* (*stx2* *lpf*) with enteroaggregative *E. coli* pathotype (*aggR aap aggA*), thereby sharing the unique virulence properties of the epidemic STEC O104:H4 strain from the international outbreak. The Polish isolates were multi-drug resistant and carried *bla*_{TEM}, *strA*, *strB*, *tetA*, *sul1* and *sul2* markers together with the *bla*_{CTX-M-15} gene for CTX-M-15 extended-spectrum β -lactamase. PFGE patterns and plasmid profiles of the Polish isolates and the epidemic STEC O104:H4 strain corresponded closely. This finding suggested an epidemiological link between the Polish STEC O104:H4 isolates and the international outbreak. Retrospective serological investigations proved person-to-person transmission of the epidemic STEC O104:H4 strain from a father who had visited Dortmund, Germany, to his 7-year-old son in Giżycko, Poland. To the best of our knowledge, this is the first report of household transmission of Shiga-toxin-producing *E. coli* in Poland.

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INTRODUCTION

Shiga-toxin-producing *Escherichia coli* (STEC) is one of the major bacterial causative agents of foodborne illness worldwide (EFSA, 2010). STEC strains produce type AB toxins which are similar to those produced by *Shigella dysenteriae*. The toxins are referred as Stx, verotoxin or Shiga-like toxin. In addition to the toxins, STEC strains often possess a large arsenal of virulence factors (for a review see Donnenberg, 2002), but Stx is arguably the most important as this toxin is responsible for manifestation of bloody diarrhoea and two severe sequelae: haemolytic uraemic syndrome (HUS) and thrombotic thrombocytopenic purpura. Consumption of contaminated food such as raw or undercooked ground meat products, raw milk, vegetables, fruits and well water is a common cause of STEC infections. Person-to-person transmission of STEC

infection has also been reported (Locking *et al.*, 2011; Rangel *et al.*, 2005).

In Europe, STEC infections are frequently reported in the UK, Germany and Sweden. In contrast, only a few cases of human STEC infections are reported annually in Poland (EFSA, 2010). All cases reported in Poland to date were sporadic, with extremely low incidence of HUS complication. Before 2011, only two confirmed cases of HUS resulting from STEC O157 and STEC O111 infections were recorded in Poland (Januszkiewicz *et al.*, 2010). Moreover, to the best of our knowledge, no STEC outbreak has been previously documented in Poland.

In early May 2011 a large foodborne outbreak caused by STEC serotype O104:H4 affected Germany then spread quickly over more than a dozen European countries. The outbreak was associated with an unusually high attack rate in female adults and a relatively high rate of HUS (approximately one HUS case per four EHEC cases) (Frank *et al.*, 2011). The *E. coli* O104:H4 outbreak lasted until

Abbreviations: ESBL, extended-spectrum β -lactamase; HUS, haemolytic uraemic syndrome; STEC, Shiga-toxin-producing *Escherichia coli*; Stx, Shiga toxin.

26 July (Robert Koch Institute, 2011a). In total, 4332 persons were affected during the outbreak. In Europe there were 852 HUS cases, including 32 deaths, and 3469 non-HUS cases, including 18 deaths (Robert Koch Institute, 2011a). The outbreak strain was found to possess an unusual combination of enteroaggregative *E. coli* (EAggEC) pathotype with StxII, which triggers HUS more frequently than StxI (Friedrich *et al.*, 2002).

In this study, we characterized two STEC O104:H4 clinical isolates collected in Poland from a household outbreak with a suspected epidemiological link to the large outbreak in Germany. We also describe a HUS case of a 7-year-old boy with insulin-dependent diabetes mellitus, who was involved in the household outbreak. To the best of our knowledge, this is the first report of household transmission of Shiga-toxin-producing *E. coli* in Poland.

METHODS

Bacterial strains and sample DNA. We tested two *E. coli* isolates, nos 158/11 and 170/11, which were, respectively, derived from stool samples of a 7-year-old boy with HUS and his 68-year-old nanny, who developed bloody diarrhoea with no HUS sequela.

A wild STEC O104:H4 strain, 155/11, that was isolated in May 2011 in Poland from a HUS case with a documented epidemiological link to the German STEC O104:H4 outbreak (Januszkiewicz *et al.*, 2011) was used as reference for molecular assays and served as a source of heat-stable antigen for ELISA. Reference *E. coli* O104:H12 (Statens Serum Institute, cat. no. 82110) was used as the source of *E. coli* O104 antigen for ELISA.

In addition to the wild STEC O104:H4 isolate 155/11, a sample of DNA from the international outbreak STEC O104:H4 strain, kindly provided by the European Union Reference Laboratory for *E. coli* (Istituto Superiore di Sanità, Rome), was used for PCR and microarray assays.

STEC O157:H7 strain EDL933 with plasmid pO157 was used as molecular standard for the multiplex PCR assay for the *stx2* and *terD* genes. EDL933 served as the negative control for the multiplex PCR assay targeting genes associated with the EAggEC phenotype.

Case description. On 1 June 2011, a 7-year-old boy with underlying insulin-dependent diabetes mellitus from Giżycko, north-eastern Poland, was admitted to Vojvodine Paediatric Hospital in Olsztyn with a 3 day history of vomiting and bloody diarrhoea. Thrombocytopenia ($60\,000$ platelets mm^{-3}) and haemolytic anaemia (haemoglobin 10.6 g dl^{-1}) were diagnosed together with initial symptoms of renal failure (urea 120 mg dl^{-1} ; serum creatinine 2.31 mg dl^{-1} ; potassium level 5.4 mmol l^{-1}). Because of the risk of HUS the boy was immediately transferred to the Department of Paediatrics and Nephrology of the Medical University in Warsaw, where HUS was confirmed. Despite supportive care, cefuroxime treatment and transfusion of fresh frozen plasma the renal failure increased over the following 48 h (serum creatinine, bilirubin and urea 6.2 , 3.6 and 296 mg dl^{-1} , respectively). The other symptoms of HUS also increased (thrombocytopenia $47\,000$ platelets mm^{-3} ; haemoglobin 9.8 g dl^{-1}). Peritoneal dialysis using a Tenckhoff catheter for 12 days in combination with rifaximin and imipenem in place of the initially administered cefuroxime improved the patient's clinical course. His renal function and thrombocytopenia improved after 20 and 15 days of hospitalization, respectively.

Eighteen days before the boy developed HUS his father had returned from Dortmund, north-western Germany, where he stayed from 9 to 14 May 2011. After his return, from 15 to 21 May the father was diarrhoeic with no complications. During his stay in Dortmund, the father ate meals with mixed salads in different restaurants without taking care whether the salads contained sprouts. He carried no sprouts or mixed salads back to Giżycko. Stool and serum samples were collected from the father after his son had been hospitalized (1 June 2011). The boy's nanny (68 years old) was admitted to a regular hospital in Giżycko on 7th June with bloody diarrhoea. No HUS sequela was diagnosed in this case. The boy and his nanny had recovered and were discharged in the last week of June. Acute and, after 2–3 weeks, convalescent phase serum samples were taken from the boy and his nanny.

Serological tests. Two different antigen preparations were used in ELISA: lipopolysaccharides (LPS) ($25\text{ }\mu\text{g ml}^{-1}$) and crude, heat-stable antigen ($10\text{ }\mu\text{g ml}^{-1}$). The LPS were extracted from the reference *E. coli* O104:H12 with trichloroacetic acid and separated by ethanol (Suzuki *et al.*, 1964). The crude antigen was extracted from the wild STEC O104:H4 strain 155/11. Strain 155/11 was cultured on Luria Broth agar at $37\text{ }^{\circ}\text{C}$ for 18 h, then the bacteria were harvested with saline (diluted to 9 MacFarland), boiled for 1 h at $100\text{ }^{\circ}\text{C}$ and stored at $-20\text{ }^{\circ}\text{C}$. ELISAs were performed based on procedures described previously (Rastawicki & Jakubczak, 2007). The result of the ELISA was taken as positive when, in the tested serum sample, antibodies were detected at the level $x + 2\text{SD}$ (where x is the arithmetic mean of the absorbance in the sera of 75 blood donors and SD is the standard deviation).

Identification and virulence analysis of STEC O104:H4.

Classical tube tests were used for *E. coli* identification (Garritty *et al.*, 2005). Resistance to tellurite and production of enterohaemolysin were determined on CT-SMAC and Enterohaemolysin blood agar, respectively (Oxoid). Production of StxII was confirmed in the two isolates tested by VTEC-RPLA assay (Oxoid). Multiplex PCR methods developed by the group of Professor Karch at the National Consulting Laboratory on HUS at the University of Münster (see http://www.ehec.org/pdf/Laborinfo_01062011.pdf; Bielaszewska *et al.*, 2011) was carried out to detect the genes *stx2*, *terD*, *rfbO104* and *fliCH4* – the key molecular hallmarks of the international outbreak STEC O104:H4 strain (Brzuszkiewicz *et al.*, 2011). The gene *stx2a* for StxIIa was detected according to WHO protocols (WHO, 2011). The EAggEC pathotype was examined by PCR-based assays according to protocols described previously (Cerna *et al.*, 2003; Czczulin *et al.*, 1999). The assays targeted the *aggR*, *aap* and *aggA* genes together with cryptic sequence AA629. The Identibac system (Alere) array EC03 with a panel of 92 virulence marker-genes commonly present in various pathotypes of *E. coli* [for details see the manufacturer's website (http://identibac.com/fileadmin/Media/Downloads/Genelist_E.coli_03_m.pdf)] was used for characterization of the virulence potential of the Polish STEC O104:H4 isolates 158/11 and 170/11.

MIC and antimicrobial resistance genes. Etest strips (bioMérieux) were used to determine susceptibility and extended-spectrum β -lactamase (ESBL) phenotype. Results were interpreted according to EUCAST or CLSI recommendations with respect to the particular antimicrobial agent (EUCAST, 2011; CLSI, 2008).

The Identibac microarray AMR05 (Alere) was used to identify the major antimicrobial resistance traits of Gram-negative bacteria (Batchelor *et al.*, 2008) in accordance with the manufacturer's protocol (see http://identibac.com/fileadmin/Media/Downloads/Genelist_AMR-ve_05_m.pdf).

The variant of the *bla*_{CTX-M} gene was determined by DNA sequencing as described previously (Dzierżanowska *et al.*, 2010).

Plasmid profiling and molecular typing. Plasmids were extracted using the NucleoBond PC 20 kit (Macherey-Nagel). Agarose gel electrophoresis (1 % agarose, Prona Plus) in Tris/acetate electrophoretic (TAE) buffer was used to separate the extracted plasmids (Sambrook *et al.*, 1989).

Pulsed-field gel electrophoresis (PFGE) was conducted on a CHEF DR II system (Bio-Rad) using *Xba*I and *Bln*I enzymes in accordance with the PulseNet protocol (Ribot *et al.*, 2006). PFGE patterns were compared using GelCompar II version 5.10 (Applied Maths).

RESULTS AND DISCUSSION

HUS cases resulting from STEC infections are rare in Poland. The case we report here was a challenge for the public health authorities in Poland, since epidemiological interview strongly suggested secondary transmission of the outbreak STEC O104:H4 strain from Germany. Three persons were involved in the household outbreak in Giżycko, Poland: the 7-year-old boy who developed HUS; his father, who had returned from Germany 2 weeks before the boy become ill; and the 68-year-old nanny of the boy.

Stool and serum samples were collected from the all three affected persons. STEC O104:H4 was isolated from the boy and his nanny. However, no STEC could be isolated from the father. Therefore, we decided to conduct serological investigations to prove person-to-person transmission of STEC serogroup O104 from the father to his 7-year-old son. The ELISA revealed high levels of specific IgA, IgG and IgM antibodies to both the *E. coli* serotype O104 LPS and the crude antigen extracted from the wild STEC O104:H4 strain 155/11 in sera obtained from the father and his son (Table 1). This finding confirmed the epidemic link between the father and his son. Moreover, IgA antibodies at the diagnostically significant level were also detected in serum samples collected from the nanny (Table 1).

Isolates 158/11 and 170/11, which were respectively collected from the boy and his nanny, were found to be tellurite-resistant, sorbitol-fermenting, non-enterohaemolytic, β -D-glucuronidase-producing *E. coli*. Production of

StxII was confirmed in these two isolates by the RPLA assay. Genetic hallmarks of the toxin (*stx2*), O104 LPS (*rfb*_{O104}) and H4 flagellar antigen (*fli*CH4) biosynthetic pathways together with the *terD* gene of the tellurite-resistance cluster were detected by multiplex PCR in both isolates. The same molecular markers were also detected in the sample DNA of the international outbreak STEC O104:H4 strain (Fig. 1a). These findings suggested that the Polish STEC isolates 158/11 and 170/11 may belong to the O104:H4 serotype.

PCR subtyping of the *stx2* gene revealed variant *stx2a* in isolates 158/11 and 170/11. The same result was observed for the sample DNA of the STEC O104:H4 international-outbreak strain (WHO, 2011). Isolates 158/11 and 170/11 were also shown by the PCR assays to carry genes *aggR*, *aap* and *aggA*, which are considered to play a key role in expression of the EAggEC pathotype (Cerna *et al.*, 2003; Czeczulin *et al.*, 1999). In addition, cryptic sequence AA629 (see Fig. 1b), also referred to as *cvd432* marker-probe (Baudry *et al.*, 1990), was detected. This cryptic sequence was reported as specific for the EAggEC phenotype. These findings together strongly suggested that the two Polish STEC isolates share key virulence properties with the German-outbreak STEC O104:H4 strain.

To gain a closer insight into the virulence potential of the tested isolates we used the Identibac system microarray EC03, which enables detection of 92 virulence markers present in common *E. coli* pathotypes. Interestingly, this assay showed that the Polish isolates were negative for the majority of the virulence markers examined. A positive spot signal was detected for only eight genes: *sigA*, *sepA*, *pic*, *lpfA*, *stx2* and *mchB*, *mchC*, *mchF*. Three of these genes, *sigA*, *sepA* and *pic*, are considered to encode serine protease autotransporters of *Enterobacteriaceae* (SPATE) (Dutta *et al.*, 2002). The *lpfA* and *stx2* genes were reported to encode long polar fimbriae and StxII, respectively. The *mch* cluster genes (*mchB*, *mchC*, *mchF*) are required for production of low-molecular-mass antimicrobial peptides secreted by members of the *Enterobacteriaceae*. These peptides are involved in microbial competition within the intestinal tract (microcins)

Table 1. Results of ELISA ($A_{450/620}$) demonstrating binding of serum antibodies to LPS *E. coli* O104 and crude heat-stable antigen of STEC O104:H4 strain 155/11

Cut-off values: 0.68 for IgA, 1.00 for IgG and 0.95 for IgM in the case of LPS antigen; 0.68 for IgA, 1.00 for IgG and 0.95 for IgM in the case of heat-stable antigen.

Patient	Age (years)	Serum sample	LPS antigen			Heat-stable antigen		
			IgA	IgG	IgM	IgA	IgG	IgM
Son	7	Acute	1.70	1.68	1.42	1.70	1.44	1.48
		Convalescent	0.98	1.10	0.56	1.00	1.15	0.64
Father	37	Acute	1.54	1.16	1.06	1.54	1.38	1.25
		Convalescent	1.25	1.10	1.15	1.32	1.20	1.10
Nanny	68	Acute	1.40	0.61	0.55	1.50	0.76	0.88
		Convalescent	1.10	0.46	0.46	1.18	0.62	0.72

Fig. 1. Electrophoretic patterns. (a) Multiplex PCR for the *stx2*, *terD*, *rfbO104* and *fliCH4* genes. (b) Multiplex PCR for the EAggEC pathotype. (c) Plasmid profiles. Plasmid size is shown in kb; Chr, fragmented chromosome and linear dsDNA. (d, e) PFGE profiles with endonuclease *XbaI* and *BlnI*, respectively. Lanes: 1, wild STEC O104:H4 strain 155/11; 2 and 3, STEC O104:H4 clinical isolates 158/11 and 170/11, respectively; 4, DNA sample of the epidemic STEC O104:H4 strain; 5, STEC O157:H7 strain EDL933. M1, GeneRuler 100 bp DNA Ladder (Fermentas); DNA size is shown in bp. M2, DNA ladder – *HindIII* digest of lambda phage; DNA size is shown in kb. M3, PFGE profiles of *Salmonella* Braenderup digested by the appropriate endonuclease. The figure was digitally edited to enhance picture quality.

(Vassiliadis *et al.*, 2010). All of these eight genes were also found in the German outbreak STEC O104:H4 strain (Bielaszewska *et al.*, 2011; Scheutz *et al.*, 2011; Rohde *et al.*, 2011). It is noteworthy that the microarray results of the sample DNA from the international outbreak STEC O104:H4 strain were exactly the same as those of the Polish isolates. The negative results for the majority of virulence markers screened by the microarray (including *eae*, *ehly*, *astA*, *subA*, *saa*, *sta1A* and *sta1B*) closely correspond with the virulence characteristics of the German outbreak STEC O104:H4 strain reported elsewhere (Bielaszewska *et al.*, 2011; Scheutz *et al.*, 2011). These findings together may suggest that the investigated Polish STEC O104:H4 isolates and the German-outbreak strain share the same virulence characteristics.

STEC O104:H4 isolates related to the German-originated worldwide outbreak were found to be multidrug resistant due to production of CTX-M-15 ESBL (Scheutz *et al.*, 2011; Bielaszewska *et al.*, 2011). The Polish STEC O104:H4 isolates 158/11 and 170/11 were resistant to ampicillin ($>256 \mu\text{g } \mu\text{l}^{-1}$), amoxicillin and clavulanic acid ($12 \mu\text{g } \mu\text{l}^{-1}$), cefotaxime ($>32 \mu\text{g } \mu\text{l}^{-1}$), ceftazidime ($12 \mu\text{g } \mu\text{l}^{-1}$), streptomycin ($192 \mu\text{g } \mu\text{l}^{-1}$), nalidixic acid ($48 \mu\text{g } \mu\text{l}^{-1}$), tetracycline ($32 \mu\text{g } \mu\text{l}^{-1}$) and cotrimoxazole

($>32 \mu\text{g } \mu\text{l}^{-1}$), and susceptible to imipenem ($0.19 \mu\text{g } \mu\text{l}^{-1}$), ciprofloxacin ($0.94 \mu\text{g } \mu\text{l}^{-1}$), chloramphenicol ($6 \mu\text{g } \mu\text{l}^{-1}$) and gentamicin ($0.75 \mu\text{g } \mu\text{l}^{-1}$). The double-disc phenotypic test showed that the both isolates produced ESBL. To identify antimicrobial resistance determinants in the tested isolates we used the Identibac system Microarray AMR05 (Batchelor *et al.*, 2008), which enables identification of major resistance genes of Gram-negative bacteria. Both Polish STEC O104:H4 isolates were shown by the array to bear genes *bla*_{TEM} and *bla*_{CTX-M}, which encode TEM-type β -lactamase and CTX-M type ESBL, together with resistance genes for streptomycin (*strA*, *strB*), tetracycline (*tetA*), sulfonamides (*sul1*, *sul2*), and the *int11* gene specific for class I integrase. DNA sequencing of the *bla*_{CTX-M} gene revealed that both the Polish STEC O104:H4 isolates carried the *bla*_{CTX-M-15} gene for CTX-M-15. Taken together, the antimicrobial resistance profile of the tested isolates corresponds closely with the profile of the epidemic STEC O104:H4 strain from the outbreak in Germany (Mellmann *et al.*, 2011; Rohde *et al.*, 2011).

The epidemic STEC O104:H4 strain from the outbreak in Germany was reported to bear two large plasmids of 75 kb and 88 kb (Rasko *et al.*, 2011). Therefore we examined the Polish STEC O104:H4 isolates for the presence of large

plasmids. Isolates 158/11 and 170/11 both harboured two large plasmids together with a ~1.3 kb small plasmid (Fig. 1c). The estimated size of the large plasmids was ~80 and ~90 kb when compared to 93 kb plasmid extracted from reference EDL933 *E. coli* O157:H7 strain (Makino *et al.*, 1998). It is noteworthy that the same plasmid profile was found in the wild STEC O104:H4 strain 155/11 (Fig. 1c) (Januszkiewicz *et al.*, 2011). In conclusion, the plasmid profile of the Polish STEC O104:H4 isolates strongly resembles that reported for the German STEC O104:H4 strain, which was also reported to bear a small plasmid of ~1.5 kb (Mellmann *et al.*, 2011; Rohde *et al.*, 2011).

To determine whether STEC O104:H4 isolates 158/11 and 170/11 of the two patients from Giżycko are clonal we genotyped the two isolates using PFGE with *Xba*I and *Bln*I. The wild STEC O104:H4 strain was also included in the PFGE typing. The *Bln*I-PFGE profiles of the three tested isolates were indistinguishable (Fig. 1e). With the exception of a single band, the same result was observed for *Xba*I-PFGE (Fig. 1d). Moreover, the *Xba*I-PFGE profiles of the Polish STEC O104:H4 isolates closely resembled the PFGE pattern of the German outbreak STEC O104:H4 strain presented on the Robert Koch Institute (Berlin, Germany) website and published elsewhere (Scheut *et al.*, 2011). The PFGE typing proved that the Polish STEC O104:H4 isolates are highly clonal and belong to the epidemic STEC O104:H4 clone from Germany.

The STEC O104:H4 strain from the German outbreak is considered an unusual HUS agent because of its antigenic structure, disputable animal origin, EAEC-type chromosomal backbone, and high pathogenicity (also referred as hypervirulent) (Mellmann *et al.*, 2011). Moreover, this strain was reported to spread effectively via direct (person-to-person) transmission in Germany as well in neighbouring countries (Kuijper *et al.*, 2011; Aldabe *et al.*, 2011; Hauri *et al.*, 2011). Compared to other European countries, Poland was relatively unaffected by the STEC O104:H4 outbreak.

To the best of our knowledge, three confirmed cases and one probable case of STEC O104:H4 infection have been reported in Poland to date. Interestingly, none of these four cases was related to consumption of contaminated food imported from Germany. The first confirmed case in Poland was a female patient of Polish nationality, who had been living in Germany for a few months. This patient had arrived at Szczecin, Poland, to meet her family then developed bloody diarrhoea that was further complicated by HUS. STEC O104:H4 isolate 155/11, referred in this study as a wild epidemic strain, was isolated from this patient (Januszkiewicz *et al.*, 2011). The second case, the adult male patient described herein, was classified as a probable case since no STEC could be isolated from this patient. The male patient had visited north-western Germany then returned to Giżycko, Poland, where he infected two persons from the same house: his 7-year-old son, who developed HUS, and the 68-year-old nanny. Both

these cases were confirmed by isolation of STEC O104:H4. A chain of diarrhoea onsets, which occurred in the following chronological order – the male patient, his son and the nanny (15 May, 29 May and 7 June 2011, respectively) – may argue for person-to-person transmission of STEC O104:H4 rather than a foodborne outbreak. It is noteworthy that the chain of infected persons reflects the intensity of their interpersonal relations: father–son and son–nanny. Among the three cases reported herein, the father was the only person who was subjected to the risk of STEC O104:H4 infection. He spent 5 days in Dortmund, North Rhine-Westphalia, a week before the peak of the STEC O104:H4 infections in Germany. The reported cumulative incidence of EHEC in North Rhine-Westphalia (1.45 per 100 000 inhabitants) was twice as high as in southern Germany (Robert Koch Institute, 2011b).

In this study we describe molecular investigations to confirm secondary transmission of the epidemic STEC O104:H4 strain from Germany to Giżycko, Poland. Although we were unable to isolate the epidemic strain from the male patient who had carried this strain from Germany, we present various molecular and serological evidence strongly arguing that the reported household outbreak in Giżycko was caused by the German STEC O104:H4 epidemic strain. We found the PCR-based molecular assays used in the reported investigations very helpful for identification and characterization of STEC O104:H4 strains related to the German outbreak.

The molecular evidence we present in this study is coherent with the aforementioned epidemiological data, and supports the initial suspicion that the epidemic STEC O104:H4 strain was transmitted by the father from Dortmund, Germany, to his son in Giżycko, Poland, and then to the boy's nanny.

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