Carriage of extended-spectrum β-lactamases in pig farmers is associated with occurrence in pigs

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Abstract

Livestock may serve as a reservoir for extended-spectrum β-lactamase-producing *Enterobacteriaceae* (ESBL-PE). The objectives of this study were to determine the prevalence of carriage with ESBL-PE in pig farmers, family members and employees, and its association with carriage in pigs. Rectal swabs were taken from 2388 pigs (398 pooled samples) on 40 pig farms and faecal samples were obtained from 142 humans living or working on 34 of these farms. Presence of ESBL-PE was determined by selective plating (agar). ESBL genes were analysed by PCR or microarray analysis, and gene sequencing. Genotypes and plasmids were determined by multilocus sequence typing and PCR-based replicon typing for selected isolates. ESBL genes were detected in *Escherichia coli* from eight humans (6%) ($bla_{CTX-M-1}$, $n = 6$; bla_{TEM-52}, $n = 1$ and $bla_{CTX-M-14}$, $n = 1$) on six farms. In 157 pig isolates (107 pooled samples) on 18 farms (45%) ESBL genes were detected ($bla_{CTX-M-1}$, $n = 12$; bla_{TEM-52}, $n = 6$; and $bla_{CTX-M-14}$, $n = 3$). Human and pig isolates within the same farm harboured similar ESBL gene types and had identical sequence and plasmid types on two farms (e.g. *E. coli* ST-453, $bla_{CTX-M-1}$, IncI1), suggesting clonal transmission. For the remaining farms, sequence types, but not plasmid types, differed. Human ESBL carriage was associated with average number of hours working on the farm per week (OR = 1.04, 95% CI 1.02–1.06) and presence of ESBLs in pigs (OR = 12.5, 95% CI 1.4–111.7). Daily exposure to pigs carrying ESBL-PE is associated with ESBL carriage in humans.

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Introduction

In Europe, an elevated prevalence of $bla_{CTX-M-1}$ has been reported in poultry, cattle and pigs [1]. Extended-spectrum β-lactamases (ESBLs) have been detected in meat samples and transmission of ESBLs from livestock to humans through the food chain has been suggested [2–5]. Antimicrobial-resistant *Enterobacteriaceae* can also be transmitted from live animals to humans [6], but evidence for a role of direct contact with livestock in human ESBL carriage is limited. Transmission of $bla_{CTX-M-1}$ harbouring IncI1 plasmids between pigs and farmers was suggested in a molecular study on two pig farms [7]. In another study, ESBL genes were detected in broiler isolates from all 26 farms studied and in isolates from six of 18 broiler farmers. Genetic similarities in genes and plasmids among isolates from farmers and broilers were documented on two farms [8]. ESBL carriage in farmers in that study (33%) is high compared with hospital patients in the southern part of the Netherlands (approximately 5%) [3]. It is also high when compared with ESBL carriage in people living in areas with high (3.6%) or low (6.7%) broiler densities in the Netherlands [9].

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Direct contact with livestock may be an important risk factor for human ESBL carriage. The objectives of this study were to determine prevalence of carriage of ESBL-producing *Enterobacteriaceae* (ESBL-PE) in pig farmers, family members and employees, and to determine the association between ESBL carriage in humans and pigs.

Materials and methods

Study design

This is a cross-sectional analysis of samples that were derived in a longitudinal study. On 40 multiplier pig farms (sows and piglets present), with or without finishing pigs, faecal samples from farmers, family members and employees were collected using faeces cups (Minigrip®) and sent to the laboratory by mail between March and October 2011. On each farm, rectal samples from 60 pigs were collected by the farm veterinarian, using sterile cotton-wool swabs (Cultiplast®) and sent refrigerated to the laboratory by courier. All animal age groups present were sampled (sows, gilts, suckling piglets, weaning piglets and finishing pigs). Rectal swabs were combined in ten pools of six pigs. Each pool consisted of an age group in the same pen. Participants filled out questionnaires on general characteristics, farm activities, and intensity and duration of animal contact. Questionnaires on farm characteristics were filled out by veterinarians and farmers. The Medical Ethical Committee of the University Medical Centre Utrecht approved the study protocol (no. 10-471/K). All participants gave written informed consent.

Laboratory analysis

Pooled swabs from pigs and faecal samples from humans were analysed for the presence of ESBL-PE by selective plating. Samples were suspended in 10 mL peptone water and incubated overnight at 37°C. For screening of ESBL-PE, suspensions were cultured on selective agar plates (*Brilliance*™ ESBL Agar, Oxoid, Basingstoke, UK) and incubated overnight at 37°C. When no growth was seen, plates were incubated for another night at 37°C. Morphologically different colonies suspected of ESBL production were cultured individually on a blood agar plate (Oxoid) and incubated overnight at 37°C. In the case of morphological uncertainty, an oxidase test was performed before culturing. Bacterial species identification of the isolates was performed by matrix-assisted laser desorption/ionization/time-of-flight (Bruker, Billerica, MA, USA). For phenotypical confirmation of ESBL-PE, a 0.5 McFarland suspension was inoculated on a Mueller–Hinton agar and a combination disc test (Rosco, Taastrup, Denmark) including cefotaxime, cefotaxime+clavulanate, ceftazidime, ceftazidime+clavulanate, cefepime and cefepime+clavulanate (*Neo-Sensitabs*™) was used to confirm the presence of ESBL-PE (EUCAST guidelines, 2012). Isolates were stored at −80°C before molecular analysis.

DNA was isolated using UltraClean® Microbial DNA Isolation Kit (MO BIO Laboratories, Inc., West Carlsbad, CA, USA). A *bla*$_{CTX-M}$ group-1-specific PCR was used to detect the presence of the most prevalent ESBL gene group [10]. DNA from *bla*$_{CTX-M}$ group-1-positive isolates was sequenced using the same primers to determine the *bla*$_{CTX-M}$ group 1 gene type. Isolates with a negative *bla*$_{CTX-M}$ group-1-specific PCR result were analysed using ESBL microarray (Check-MDR CT101, Checkpoints, Wageningen, the Netherlands) to detect *bla*$_{TEM}$ group, *bla*$_{SHV}$ group or another *bla*$_{CTX-M}$ group (2,8,9,25). DNA from ESBL microarray-positive isolates was sequenced with group-specific primers to determine the exact gene type [11,12]. DNA sequences were interpreted with Basic Local Alignment Search Tool (National Center for Biotechnology Information). For *bla*$_{SHV}$ and *bla*$_{TEM}$ groups, sequences were also aligned with non-ESBL types (*bla*$_{TEM-1}$, *bla*$_{SHV-1}$) to determine the exact gene type based on single nucleotide polymorphisms.

Genotyping and plasmid characterization were performed for human and pig isolates with similar ESBL genes identified in the same farm. For each human isolate, one pig isolate from the same farm was selected. Genotypes and plasmid types were determined by multilocus sequence typing (MLST) (MLST databases at UoW, Warwick, UK) and PCR-based replicon typing, respectively [13]. The location of the ESBL genes on plasmids was tested by transformation. *Escherichia coli* cells (ElectroMAX DH10B™ cells, Life Technologies, Paisley, UK) were used as recipient and Luria–Bertani agar plates containing cefotaxime (1 mg/L) as selective agent. Whole genome sequencing was available for eight human and pig isolates, plasmid types and sequence types (ST) were reconstructed from these data [14].

Data analysis

Farms were classified as ESBL-positive if an ESBL gene was detected in an isolate from at least one pooled pig sample. Statistical analyses were performed using SAS version 9.2 (SAS Institute Inc., Cary, NC, USA). The association between ESBL carriage in humans and pigs was calculated with logistic regression analysis (PROC GENMOD) adjusted for clustering at farm level. Presence of ESBLs in pigs, average number of hours working per week, and potential confounders age, gender and smoking were analysed univariately and selected for multivariate analysis when the p-value was <0.2. Model selection was performed by a backward procedure. Model fit was checked with the QIC-statistic (Quasi-likelihood under the Independence model Criterion). The final model retained variables significant at $p \leq 0.05$.

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TABLE 1. General characteristics of the participants of the study (n = 142)

Human characteristics	Frequency (%)
Gender	
Male	85 (60)
Female	57 (40)
Category	
Farmer	47 (33)
Family of farmer	76 (54)
Employee	19 (13)
Age (mean, range)	142 (36, range 6–79)
Age <18 years	30 (21)
Age 18–65 years	109 (77)
Age >65 years	3 (2)
Living on farm	
Yes	113 (80)
No	29 (20)
Average number of hours working on the farm per week	134 (mean 25, range 0–80)
0	42 (31)
1–20	27 (20)
>20	65 (49)
Frequency of pig contact	129
Daily	64 (50)
Weekly	25 (19)
Monthly	9 (7)
Less than monthly	31 (24)
Smoking	141
Yes	13 (9)
No	128 (91)

Results

Participant characteristics

In total, 142 people (47 farmers, 76 family members and 19 employees) living/working on 34 of the 40 farms (one to eleven persons per farm) participated (Table 1). The response rate was 68%. The main reason for non-participation was the objection to collect faecal samples.

TABLE 3. Extended-spectrum β-lactamase (ESBL) genes in pooled samples from pigs in different age groups

Age group	Pooled samples, *n*	Pooled samples with ESBL genes detected, *n* (%)
Sows	76	22 (29)
(Rearing) gilts	77	12 (16)
Suckling piglets[a]	78	23 (29)
Weaned piglets	107	29 (27)
Finishing pigs	52	15 (29)
Total	390[b]	101 (26)

[a]Suckling piglets = pooled sample contained rectal swabs from one mother sow and five of her suckling piglets.
[b]No age group was reported for eight pools in one farm. On two farms, only nine pools were analysed.

Farm characteristics and pig isolates

On average, 470 sows were present per farm (range 110–1100). Finishing pigs were present on 27 of 40 farms. Farms without finishing pigs either supplied pigs for finishing pig farms (*n* = 11) or supplied gilts for other multiplier farms (*n* = 2). On 23 farms gilts were supplied by another farm, the other 17 farms had their own gilt supply (closed farms).

From 40 farms, 398 pooled samples (ten pooled samples per farm, six rectal swabs each; on two farms, only nine pools were analysed) taken from 2388 pigs were analysed. In total, 168 pig isolates (114 pooled samples) from 23 farms were phenotypically confirmed ESBL-PE (with combination disc test) (*E. coli* (*n* = 160), *Citrobacter freundii* (*n* = 4), *Enterobacter cloacae*

TABLE 2. Extended-spectrum β-lactamase (ESBL) genes in 157 pig and 12 human isolates

Pig isolates					Human isolates	
Farm	Positive pooled samples, *n*	ESBL type 1, isolates (*n*)	ESBL type 2, isolates (*n*)	ESBL type 3, isolates (*n*)	Person	Gene type, isolates (*n*)
1	9/10	$bla_{\text{CTX-M-1}}$ (16)[a]				
2	10/10	$bla_{\text{CTX-M-1}}$ (16)				
3	9/10	$bla_{\text{CTX-M-1}}$ (13)			3a	$bla_{\text{CTX-M-1}}$ (1)
4	5/10	$bla_{\text{CTX-M-1}}$ (7)[b]				
5	5/10	$bla_{\text{CTX-M-1}}$ (7)			5a	$bla_{\text{CTX-M-1}}$ (1)
					5b	$bla_{\text{CTX-M-1}}$ (1)
6	4/9	$bla_{\text{CTX-M-1}}$ (4)				
7	1/10	$bla_{\text{CTX-M-1}}$ (2)[c]				
8	8/10	$bla_{\text{CTX-M-1}}$ (11)	$bla_{\text{TEM-52}}$ (2)			
9	8/10	$bla_{\text{CTX-M-1}}$ (10)	$bla_{\text{CTX-M-32}}$ (1)		9a	$bla_{\text{CTX-M-1}}$ (1)
					9b	$bla_{\text{CTX-M-1}}$ (1)
10	5/10	$bla_{\text{TEM-52}}$ (9)				
11	1/10	$bla_{\text{TEM-52}}$ (2)[d]				
12	1/10	$bla_{\text{TEM-52}}$ (1)				
13	8/10	$bla_{\text{TEM-52}}$ (13)	$bla_{\text{CTX-M-1}}$ (1)	bla_{TEM} group 3 (1)[e]	13a	$bla_{\text{TEM-52}}$ (2)
14	9/10	$bla_{\text{TEM-52}}$ (6)	$bla_{\text{CTX-M-2/97}}$ (4)	$bla_{\text{CTX-M-1}}$ (1)		
15	6/10	$bla_{\text{CTX-M-14}}$ (7)			15a	$bla_{\text{CTX-M-14}}$ (2)
16	1/10	$bla_{\text{CTX-M-14}}$ (1)				
17	8/10	$bla_{\text{CTX-M-14}}$ (10)	$bla_{\text{CTX-M-1}}$ (1)			
18	9/10	$bla_{\text{CTX-M-15}}$ (11)				
19	0/10				19a	$bla_{\text{CTX-M-1}}$ (3)

[a]CTX-M-1 isolates were not tested for additional genes.
[b]Six isolates were *Escherichia coli* and one isolate was *Escherichia* sp.
[c]One isolate was *E. coli* and one isolate was *Salmonella* sp.
[d]One isolate was *E. coli* and one isolate was *Escherichia fergusoni*.
[e]Further specification by sequence analysis was unsuccessful.

TABLE 4. Sequence types of Escherichia coli isolates and plasmid characterization

Farm	Origin	Gene type	*E. coli* ST	Plasmid type
3	Human	$bla_{CTX-M-1}$	ST-540*	IncI1
3	Pig	$bla_{CTX-M-1}$	ST-165[SLV*†,a]	IncI1
5	Human	$bla_{CTX-M-1}$	ST-453*	IncI1*
5	Human	$bla_{CTX-M-1}$	ST-453*	IncI1*
5	Pig	$bla_{CTX-M-1}$	ST-453*	IncI1
5	Pig	$bla_{CTX-M-1}$	ST-453*	IncI1
9	Human	$bla_{CTX-M-1}$	ST-711*	IncI1*
9	Human	$bla_{CTX-M-1}$	NT[†,b]	IncI1, NTR[c]
9	Pig	$bla_{CTX-M-1}$	ST-3321*	IncI1*
9	Pig	$bla_{CTX-M-1}$	ST-711	IncI1
13	Human	bla_{TEM-52}	ST-10	IncI1, NTR
13	Pig	bla_{TEM-52}	NT[†,d]	IncI1
15	Human	$bla_{CTX-M-14}$	ST-3079	ColIE2
15	Pig	$bla_{CTX-M-14}$	ST-744[SLV†,e]	ColIE2, NTR

*Data constructed from whole genome sequencing data [14]; †allele codes are available as supportive information.
[a]Single locus variant of ST-165.
[b]New type, not registered yet.
[c]No successful transformation.
[d]New type, not registered yet. Not similar to other new type found.
[e]Single locus variant of ST-744.

(*n* = 1), *Salmonella* sp. (*n* = 1), *Escherichia* sp. (*n* = 1) and *Escherichia fergusoni* (*n* = 1)). ESBL genes were detected in 157 isolates (107 pooled samples) from 18 farms (45%) (*E. coli* (*n* = 154), *Salmonella* sp. (*n* = 1), *Escherichia* sp. (*n* = 1) and *E. fergusoni* (*n* = 1)). Eleven phenotypically confirmed ESBL-PE isolates did not harbour one of the tested ESBL genes (*E. coli* (*n* = 6), *Citrobacter freundii* (*n* = 4), *Enterobacter cloacae* (*n* = 1)). $bla_{CTX-M-1}$ was detected most frequently (12 farms, 89 isolates (57%)), followed by bla_{TEM-52} (6 farms, 33 isolates (21%)) and $bla_{CTX-M-14}$ (3 farms, 18 isolates (11%)) (Table 2). ESBL gene presence in pooled samples ranged from 16% in (rearing) gilts to 29% in sows, suckling piglets and finishing pigs (Table 3).

Human isolates

From 142 humans, 16 isolates from 12 people were phenotypically confirmed as ESBL-PE (*E. coli* (*n* = 13), *C. freundii* (*n* = 1), *Enterobacter cloacae* (*n* = 1), and *Proteus vulgaris* (*n* = 1)). ESBL genes were detected in 12 isolates (all *E. coli*) from eight people (6%). $bla_{CTX-M-1}$ was found in isolates from six participants, the other two participants carried bla_{TEM-52}-positive and $bla_{CTX-M-14}$-positive isolates, respectively. Five out six humans who carried $bla_{CTX-M-1}$ were living/working on a farm where $bla_{CTX-M-1}$ was exclusively (*n* = 3) or predominantly (*n* = 2) detected in pig isolates. The farmer who carried bla_{TEM-52} worked on a farm where bla_{TEM-52} was predominantly detected in pig isolates and the farmer who carried $bla_{CTX-M-14}$ worked on a farm where $bla_{CTX-M-14}$ was exclusively detected in pig isolates (Table 2).

Prevalence of human ESBL carriage was 6% (95% CI 2–9%). The prevalence among 64 individuals with daily pig contact was 11% (95% CI 3–19%) and among 52 individuals living/working on a farm with ESBL-PE carrying pigs prevalence was 13% (95% CI 4–23%). Prevalence was highest among 26 individuals with daily pig contact on a farm where ESBL genes were detected in pigs; 27% (95% CI 10–44%).

TABLE 5. Univariate and multivariate analyses for extended-spectrum β-lactamase (ESBL) carriage in farmers, family members and employees

Determinant	Univariate analysis OR	95% CI	p	Multivariate analysis OR	95% CI	p
Age	1.05	>1.00–1.09	0.01			
per 10 years	1.7	1.1–2.5	0.01			
Gender (male vs. female)	2.4	0.6–10.1	0.23			
Smoking (yes vs. no)	1.3	0.1–12.2	0.80			
Average number of hours working on farm per week	1.04	1.02–1.06	0.0001	1.04	1.02–1.06	0.0008
per 10 hours	1.5	1.2–1.8	0.0001	1.5	1.2–1.8	0.0008
Presence of ESBLs in pigs (yes vs. no)	13.9	1.7–115.0	0.01	12.5	1.4–111.7	0.02

MLST and plasmid analyses

Bacterial strain typing by MLST and plasmid replicon typing were performed on seven human isolates and seven pig isolates from five farms (Table 4). Three sets of human and pig isolates were identical in ST and plasmid type. For example in farm 5, two human and two pig isolates were identified as *E. coli* ST-453, harbouring $bla_{CTX-M-1}$ on an IncI1 plasmid. In the remaining four pairs, plasmid types but not STs were identical. In three pairs, transformation did not succeed in one isolate.

Association between ESBL carriage in humans and pigs

ESBL carriage was detected in seven of 52 humans living/working on farms where ESBL genes were detected in pigs and in one of 90 humans living/working on farms where ESBL genes were not detected in pigs (OR = 13.9, 95% CI 1.7–115.0, p 0.01) (Table 4). When this analysis was restricted to isolates harbouring $bla_{CTX-M-1}$, the association remained (OR = 17.9, 95% CI 1.9–168.2, p 0.01). As the prevalence is too low to perform separate analyses for each gene type, no distinction in ESBL gene types is made in further analyses. ESBL carriage in humans was associated with average number of hours working on the farm per week (OR = 1.04, 95% CI 1.02–1.06, p 0.0001), and age (OR = 1.05, 95% CI > 1.00 to 1.09, p 0.01). All carriers (six farmers and two family members) reported to work at least 20 hours per week on the farm. All seven carriers that lived on a farm with ESBL-PE-carrying pigs reported to have daily pig contact. No differences in prevalence could be estimated for frequency of pig contact, living on farm, and being a farmer, family member or employee because there was little or no variation within the group of carriers. Results of univariate analyses are presented in Table 5.

Age, average number of hours working on the farm per week, and presence of ESBLs in pigs were selected for multivariate analysis. Human carriage of ESBLs was positively associated with average number of hours working on farm per week (OR = 1.04, 95% CI 1.02–1.06, p 0.0008), and presence of ESBLs in pigs (OR = 12.5, 95% CI 1.4–111.7, p 0.02). The final model is presented in Table 5.

Discussion

Among people working/living on pig farms, frequent contact with pigs carrying ESBL-PE is associated with ESBL carriage. Working hours on the farm (indicating direct contact with pigs) and presence of ESBLs in pigs on the farm were associated with human ESBL carriage. $bla_{CTX-M-1}$ was found in six of eight humans and 12 of 18 farms.

The overall prevalence for human ESBL carriage was 6% (95% CI 2–9%), comparable to the reported prevalence of 5% in a hospital patient population in the Netherlands [3], and to ESBL carriage in people living in Dutch municipalities with high or low broiler densities (3.6 and 6.7%, respectively) [9]. In the current study, the prevalence was 13% (95% CI 4–23%) among people living/working on a farm with ESBL-PE-carrying pigs, and 27% (95% CI 10–44%) among humans with daily exposure to ESBL-PE-carrying pigs. The latter is comparable to the prevalence among 18 Dutch broiler farmers (33%) working on farms where ESBL genes were detected in broilers [8].

ESBL gene types detected in humans corresponded to those exclusively or predominantly detected in pigs on the respective farms. These results are suggestive for transmission of ESBL genes from pigs to humans (or vice versa). The similarity in ST and plasmid type between human and pig isolates found on two farms strongly suggests the occurrence of clonal transmission. This is endorsed by whole genome sequencing results; on farm 5, only six single nucleotide polymorphisms were found between one farmer isolate and two related pig isolates [14]. Results from other studies suggest that spread of ESBL genes in *E. coli* between animals and farmers predominantly results from horizontal dissemination of plasmids, rather than from transmission of bacterial strains [7,8].

High use of antimicrobials in livestock production is considered to be associated with a high prevalence of ESBL-PE in animals [15]. Antimicrobial use in pigs on these farms is comparable to the use in the Dutch pig farm population [16].

Faecal samples were collected through self-sampling and sent to the laboratory by mail. As *E. coli* is known to survive in faeces for a longer period without refrigeration, it is unlikely that this led to under-detection of ESBL-PE [17,18].

After molecular analysis, ESBL genes were determined in 169/184 (92%) of the phenotypically determined ESBL-PE (both human and pig isolates), which is in line with the positive predictive value of 93% for local ESBL confirmation found in a multi-centre evaluation study [19]. In the present study, most *Enterobacteriaceae* other than *E. coli* with ESBL phenotype did not harbour ESBL genes. In four of 15 isolates, the presence of AmpC β-lactamases was confirmed with the ESBL microarray used (data not shown). Inducible chromosomal AmpC is often present in *Enterobacteriaceae* other than *E. coli* and *Klebsiella pneumoniae* and the presence of chromosomal or plasmid mediated AmpC β-lactamases or other β-lactamases might partially explain the discrepancy [19–23]. AmpC β-lactamases were not further explored in this study. Isolates with PCR-confirmed bla_{CTX-M} group 1 were not further analysed with the ESBL-microarray. This may have resulted in under-reporting of other ESBL genes present in the same isolate. Yet, the likelihood of multiple different ESBL genes in a single isolate is considered to be low [4].

Hospitalization and foreign travel are known risk factors for human carriage of ESBLs [24,25]. None of the eight ESBL carriers had been hospitalized or had used antibiotics in the preceding 12 months. Five ESBL carriers had been abroad in the preceding 12 months (France (n = 3), Scotland (n = 1), Turkey (n = 1)), and travelling data were missing from three ESBL carriers. ESBL-PEs have been reported widely in livestock and meat [1–5,26], but they have also been found in healthy and diseased companion animals [27,28], wildlife [29], vegetables [30], drinking water [31] and sewage [30]. Duration of human ESBL carriage can be prolonged [32,33], although determinants influencing duration of carriage are not well understood. In the current cross-sectional study, duration of ESBL carriage was not determined, but a clear association between human ESBL carriage and direct contact with ESBL-PE carrying pigs was seen. Together with the increasing occurrence of ESBLs in livestock worldwide, livestock farmers appear to have a higher risk of carrying ESBLs than members of the general population. In this study we did not observe human ESBL carriers without frequent exposure to pigs. More research is needed on duration and risk of ESBL carriage. Moreover, the attributable role of ESBL carriage in farmers for public health remains unclear until more knowledge is gained on the transmission of ESBLs from farmers into the community.

Conclusion

Carriage of ESBL in people working/living on pig farms is associated with frequent contact with pigs carrying ESBL-PE. In both humans and pigs, $bla_{CTX-M-1}$ was the most frequently

detected ESBL gene and genetic similarities were seen in plasmid types and STs.

Transparency declarations

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