

Original Article

Serologic evidence of *Echinococcus granulosus* in slaughterhouses in Pakistan: global alarm for butchers in developing countries

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Abstract

Introduction: Cystic echinococcosis, caused by *Echinococcus granulosus*, is a neglected zoonosis that affects humans and livestock. This sero-survey was designed for the first time in Pakistan to assess the exposure of butchers to *E. granulosus* as there was no previous report in the country for this occupational group.

Methodology: Blood samples were collected from registered butchers (n = 364) in five different slaughterhouses in Faisalabad and Bahawalnagar Districts. Sera were tested for anti-*Echinococcus granulosus* IgG with a commercially available ELISA kit (specificity, 100%; sensitivity, 97%).

Results: Overall, seroprevalence was 9.61% (35/364). Butchers >30 years of age (10.34%), those involved in small ruminants butchery (11.70%), >10 years' experience (10.04%), formal education level up to middle standard (10.28%), contact with dogs (12.71%), improper/unhygienic disposal of dog feces (11.87%), and those unaware of the consequences of eating with unwashed hands (13.80%) were more seropositive with significant statistical differences ($p < 0.05$). Variables like previous cyst encounter, no knowledge of zoonoses and/or cystic echinococcosis, living in rural areas and the presence of stray/feral dogs in surroundings did not show any significant association ($p > 0.05$) with seroprevalence in butchers. The binary logistic regression model also showed a statistically significant relationship ($p < 0.05$) for all risk factors found statistically significant ($p < 0.05$) in the univariate analysis.

Conclusions: This study shows high prevalence of cystic echinococcosis among butchers in Pakistan and underscores the need for educating native slaughterhouse personnel on cystic echinococcosis. It also serves as a global warning, especially in developing countries.

Key words: Butchers; ELISA; *Echinococcus granulosus*; occupational risk, Pakistan; seroepidemiology.

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Introduction

Cystic echinococcosis (CE) is a neglected tropical disease (NTD) caused by the metacestode larval stage of *Echinococcus granulosus* [1,2] that requires two hosts for the completion of its life-cycle. Canids (both domestic and wild) serve as the definitive host that harbors the adult worms in the intestine and responsible for the release of eggs that are infective to intermediate hosts (sheep, goats, cattle, camels, etc.) while grazing on contaminated pastures [3]. Eggs hatch in the intestine of herbivorous intermediate hosts, penetrates the intestinal lining and it's subsequently distributed throughout the body after gaining access to the

circulatory system. The liver and lungs are the most frequent predilection sites where the developing embryos transform into hydatid cysts containing thousands of protoscolices [4]. Humans become infected by accidental ingestion of eggs/proglottids through consumption of contaminated vegetables and fruits or water leading to the development of cysts in different organs [5–7]. Another possible way of acquiring the infection is by direct contact with feces of infected definitive hosts [8].

Human CE runs in chronic course virtually with asymptomatic form. The development of pathologic lesions (cysts) determines the severity score and

prognosis of the disease [9]. In most human cases, the liver is the predominant site (70%) for cyst development followed by lungs (20%) and other visceral organs (about 10%) [10]. Hepatic CE patients exhibit mild to severe abdominal pain and sensation of abdominal mass [11].

CE occurs globally, however, China, India, Russia, Middle-east, New Zealand, Australia, South America, France and North Africa are main endemic areas [12]. Epidemiological investigations reveal high echinococcosis endemicity in these countries, with heavy economic and social setbacks [13–16]. The annual incidence of CE in humans is reported to be around 1 million with expenses amounting to \$ 3 billion every year [17].

Several practices that favor the transmission of *E. granulosus* lead to a high prevalence of CE in different parts of the world. For example, contact with dogs, home slaughtering practices without proper supervision by trained veterinarians, and feeding of raw offal to dogs [18]. Construction of slaughterhouses around human settlements with poor infrastructures, lack of community awareness on CE and stray dog access to CE condemned carcasses through indiscriminate disposal of abattoir waste also contribute to the transmission of CE within a community [19].

In view of the endemic status of CE in contiguous countries, slaughterhouse malpractices and poor facilities, and substantial economic damages, this preliminary survey was aimed to report the seroprevalence of CE in butchers (responsible for killing and slaughtering animals commercially) in slaughterhouses located in different prefectures of Punjab province of Pakistan and to ascertain the associated risk factors.

Table 2. Risk factors for cystic echinococcosis (CE) investigated in the study.

Sr. No.	Variable
1	Age (> 30 years; ≤ 30 years)
2	Working experience (> 10 years; ≤ 10 years)
3	Type of animal slaughtered (small ruminants; large ruminants)
4	Literacy level (up to 8th standard; more than 8th standard)
5	Living setup (rural; urban)
6	Cyst knowledge (yes; no)
7	Heard of zoonoses (yes; no)
8	Heard of CE (yes; no)
9	Presence of stray dogs in vicinity (yes; no)
10	Association with dogs (yes; no)
11	Contact with dogs feces and disposal (yes; no)
12	Awareness on eating contaminated food items (yes; no)

Table 1. Number of butchers registered at various slaughterhouses selected in the study.

Slaughterhouse location	Number of registered butchers
Jaranwala	51
Gojra	52
Samundari	48
Bahawalnagar	182
Tandlianwala	31

Methodology

Ethical approval

The study was approved and supervised by the Laboratory of Preventive Veterinary Medicine and Public Health, University of Agriculture, Faisalabad, Pakistan. Written permission from the stationed Veterinary Officers of selected slaughterhouses was also taken. Informed consent was obtained from all registered personnel after they were briefed on the nature of the study and their role as participants.

Study area and sample collection

Abattoirs located in five different districts of Faisalabad and Bahawalnagar were selected with study permission obtained from the respective Municipal Corporations.

Blood samples were drawn through venipuncture aseptically from 364 butchers of different age groups in 5ml disposable syringes and were transferred into gel-clot activator serum vials (Improvacuter, Hamburg, Germany) and labeled accordingly. The numbers of registered butchers in each study area are mentioned in Table 1.

Demographic information such as age, duration in butchery, living settings (rural or urban), species of animals slaughtered, literacy level and knowledge of the disease was recorded on pre-designed questionnaires. Participants were also inquired about dog ownership as pets/shepherd dogs, dog presence in their surroundings (houses/workplaces), contact with dog feces, and possibility of eating contaminated food (Table 2).

Sera were harvested upon centrifugation (4,000 rpm for 15 minutes) and stored in cryovials in a refrigerator at -20 °C until use [20].

Serological Examination (iELISA)

The investigations of antibodies (IgG) to *E. granulosus* were carried out by a commercially available indirect ELISA kit (DRG Instruments GmbH, Marburg, Germany). This kit is a solid-phase enzyme-linked immunosorbent assay with microtiter wells coated with crude *E. granulosus* antigen with

specificity and sensitivity of 100% and 97%, respectively [21,22].

The testing was carried out stringently following the manufacturer's directions. Briefly, serum samples and ready-to-use controls were pipetted into the 96-well plates. During incubation, *Echinococcus*-specific antibodies of positive specimens and controls were bound to the immobilized antigens. After a washing step to remove unbound antibodies and control material, horseradish peroxidase-conjugated anti-human IgG antibodies were dispensed into the wells. During a second incubation, this anti-IgG conjugate bound specifically to IgG antibodies resulting in the formation of enzyme-linked immune complexes. After a second washing step to remove unbound conjugate, the immune complexes formed (in case of positive results) were detected by incubation with 3,3', 5,5'-Tetramethylbenzidine TMB substrate with the development of a blue color. The blue color then turned yellow upon addition of sulfuric acid. The intensity of this color was directly proportional to the amount of *Echinococcus*-specific IgG antibody in the specimen. Absorbance at 450 nm was read using a Bio-Rad microplate reader (iMark Microplate Absorbance Reader) within 30 minutes after adding the stop solution.

Validation of test run and interpretation

The test was considered valid if absorbance values of (i) substrate blank and negative control were less than 0.100 and 0.200, respectively and (ii) absorbance values of cut-off control and positive control were between 0.350-0.850 and 0.300-0.650, respectively. The samples having absorbance values more than 10% above cut-off control were declared as positive while those with absorbance values more than 10% below cut-off control were interpreted as negative [23]. Ten diagnosis-related group (DRG) Units (DU) was taken as the threshold value and all values greater than this threshold were considered positive [22].

Statistical analysis

Prevalence was estimated at 95% confidence interval (CI) [24]. Chi-square test was applied to test the association between variables with statistical significance at $p < 0.05$. Univariate analysis was conducted by applying chi-square test and odds ratios were calculated. Finally, a binary logistic regression analysis was conducted to assess the association between seroprevalence of *E. granulosus* and variables found significant in the initial univariable screening. All tests were carried out in IBM SPSS Statistics 17.0 for Windows® (IBM Corporation, Route 100 Somers, New York, USA).

Results

Overall, 35/364 tested sera (9.61%, 95% CI = 7.00-13.08) were positive for anti-*Echinococcus granulosus* IgG. The seroprevalence ranged from 6.04% in Bahawalnagar to 25% in Samundari. The highest seroprevalence was recorded among abattoir workers from Samundri (25%) followed by Jaranwala (11.76%), Gojra (7.69%), Tandlianwala (6.45%), and Bahawalnagar (6.04%). A statistically significant difference ($p < 0.05$) in prevalence was observed in the survey areas (Table 3).

In terms of the age of butchers, those >30 years of age showed higher seroprevalence (10.34%) than ≤30 years age group (9.13%) with statistical significant difference ($p < 0.05$). Also, butchers with >10 years' experience were more seropositive (10.04%, 95% CI = 4.78-10.81) than those with ≤10 years (9.03%) slaughtering experience with statistical significance ($p < 0.05$). Furthermore, butchers slaughtering small ruminants (sheep and goats) demonstrated a higher seropositive rate (11.70%) than those engaged in large animals (8.88%) ($p < 0.05$).

Literacy level-based difference in seroprevalence was found both in numerical and statistical terms ($p < 0.05$) such that individuals with formal education up to middle standard (10.28%) were more seropositive than those having secondary education (4.65%). Other factors investigated include whether butchers have seen

Table 3. Seroprevalence of *Echinococcus granulosus* in abattoir workers in different localities of Punjab Province, Pakistan.

District	Prefecture	Positive / Tested	Prevalence % (95% CI)	p-value (χ^2)
Faisalabad	Samundari	12/48	25 (14.92-38.78)	0.0144 (12.44)
	Jaranwala	6/51	11.76 (05.50-23.38)	
	Gojra	4/52	7.69 (3.03-18.17)	
	Tandlianwala	2/31	6.45 (1.79-20.72)	
Bahawalnagar	Bahawalnagar	11/182	6.04 (3.41-10.49)	
Total		35/364	9.61 (7.00-13.08)	

a cyst during slaughter prior or heard of zoonoses and/or CE. Twenty-two seropositive (10.04%) butchers had encountered a cyst, 31 (10.72%) had never heard of zoonoses, and 27 (10.71%) had no prior knowledge of CE. Slaughterhouse personnel living in rural areas demonstrated similar seroprevalence (9.67%) than those living in urban areas (9.59%) ($p > 0.05$).

Higher seroprevalence (10.70%) was also recorded in butchers who answered “yes” to stray dog presence around their vicinity but with no statistical differences ($p > 0.05$). More positive individuals were found among those who were in contact with dogs (12.71%) than those with no history of stray or domesticated dog contact (4.41%) ($p < 0.05$). A statistical significance ($p < 0.05$) in seroprevalence was also observed between butchers who frequently come in contact with dog feces (11.87%) during disposal than those who are not directly involved in the disposal (2.32%).

Further, individuals with a history of consuming edible items with unwashed hands demonstrated higher seroprevalence (13.80%) than their counterparts (3.89%) and was statistically significant ($p < 0.05$).

Univariate analysis (Table 4) revealed that butchers in > 30 years category (OR = 1.13), individuals engaged

in butchery >10 years (OR = 1.11), seen a cyst (OR = 1.12), those slaughtering small ruminants (OR = 1.32), personnel with middle education standard (OR = 2.21), never heard of zoonoses (OR = 2.01) and CE (OR = 1.50) and residing in rural setups (OR = 1.01) showed higher chances of being infected. The presence of stray dogs in the vicinity (OR = 1.66), association with dogs (OR = 2.88), contact with dog feces (OR = 5.10) and eating with unwashed hands (OR = 3.54) were other important risk factors for acquiring the infection.

All variables found significant in the univariate analysis ($p < 0.05$) were included in the final binary logistic regression analysis. However, variables like prior encounter of cysts, knowledge of zoonoses and CE, living conditions of the butchers and the presence of stray dogs around vicinities were removed from the model at subsequent steps as $p > 0.05$. Age category > 30 years, Butchers with more than 10 years’ experience, slaughtering small animals, literacy level up to middle standard, association with dogs, contact with dog feces during disposal and poor knowledge on the implications of eating with unwashed hands, showed statistical significance ($p < 0.05$) (Table 5).

Table 4. Univariate analysis of risk factors and their association with *Echinococcus granulosus* seropositivity in abattoir personnel.

Variable	Category	Positive / Tested	Prevalence % (95% CI)	OR (95% CI)	χ^2	p-value
Age	> 30 years	15/145	10.34 (6.37- 16.36)	1.13 (0.56-2.28)	16.33	0.041*
	≤ 30 years	20/219	9.13 (5.99-13.68)	-		
Working experience	> 10 years	21/209	10.04 (4.78-10.81)	1.11 (0.55-2.25)	19.44	0.019*
	≤ 10 years	14/155	9.03 (5.45-14.59)	-		
Seen cyst	Yes	22/219	10.04 (6.73-14.75)	1.12 (0.55-2.29)	14.75	0.103
	No	13/145	8.96 (5.32- 14.74)	-		
Type of animal slaughtered	Small	11/94	11.70 (6.00-19.00)	1.32 (0.62-2.78)	32.54	0.021*
	Large	24/270	8.88 (6.00-12.00)	-		
Literacy level	Up to middle (8 th)	33/321	10.28 (7.41-14.09)	2.21 (0.52-9.39)	29.39	0.039*
	Secondary (>8 th)	2/43	4.65 (12.8-15.45)	-		
Heard of zoonoses	No	31/289	10.72 (7.66- 14.83)	2.01 (0.69-5.84)	5.78	0.1932
	Yes	4/75	5.33 (2.09- 12.92)	-		
Heard of CE	No	27/252	10.71 (7.46- 15.36)	1.50 (0.66-3.39)	4.49	0.3296
	Yes	8/112	7.14 (3.66- 13.46)	-		
Living setting	Rural	9/93	9.67 (5.18-17.38)	1.01 (0.46-2.22)	17.42	0.382
	Urban	26/271	9.59 (6.63-13.68)	-		
Stray dog in vicinity	Yes	29/271	10.70 (7.55- 14.94)	1.66 (0.67-4.10)	15.77	0.592
	No	6/93	6.45	-		
Association with dogs	Yes	29/228	12.71 (9-17.67)	2.88 (1.17-7.10)	22.66	0.027*
	No	6/136	4.41 (20.4- 9.29)	-		
Contact with dog feces for disposal	Yes	33/278	11.87 (8.58- 16.2)	5.10 (1.21-21.54)	17.56	0.0146*
	No	2/86	2.32 (0.64- 8.09)	-		
Awareness about eating with unwashed hands	No	29/210	13.80 (9.79- 19.13)	3.54 (1.44-8.72)	20.73	0.0092*
	Yes	6/154	3.89 (1.8- 8.24)	-		

* significant difference ($p < 0.05$).

Discussion

Pakistan is a country with a population of about 200 million people that resides mainly in rural areas with poor social amenities. Almost 80% of Pakistani peasants directly or indirectly rely on the agriculture and livestock sectors for their livelihoods. Meanwhile, occupation remains one of the most important determinants in the epidemiology of CE disease [3].

Globally, CE poses serious social and economic threats especially in endemic areas [25]. In the last decade, there are reports of increasing CE prevalence in contrast to previous years [26] and prevalence is reportedly higher (15.43%) in occupational risk group among which abattoir workers are arguably at potential risk of being infected with *E. granulosus* [27]. Despite the socio-economic implications, CE is considered a neglected tropical zoonotic disease which indicates it receives little attention from various health departments, policy and decision-makers, the mainstream media.

In this study, we observed an overall seroprevalence of 9.61% among butchers from five locations in Pakistan. Our result conforms with previous seroprevalence observations in countries like Iran, China and Mongolia [28,31,32]. In neighboring countries like Iran, CE is a major public health concern and has been reported to be endemic particularly in Western and Northern rural Iran. A similar investigation among abattoir workers revealed a 5.5% seroprevalence in Khorasan province of Iran [28]. In this study, we found that seroprevalence differs with locations in accordance to the reports of Al-Shaibani et al. [29] who observed varying CE prevalence among different governorates of Iran where significantly higher infection rates were observed in Dhamar governorate (3.59%) than in Taiz governorate (1.90%) [29] and was possibly due to prevailing risk factors.

In Mongolia, CE also constitutes a serious public health issue and it is endemic in 12 provinces, out of which 10 share borders with Russia and China [30]. Serological testing among inhabitants of Mongolian

Dornodand Selenge and Ulaanbaatar showed that 9.1% and 8.5% of the tested inhabitants were seropositive for CE [31,32], which is in tandem with our surveillance. Besides, 5.2% of North-western Mongolians were also found to be highly seropositive [33]. CE is one of the major zoonotic threats in Central Asian Republics (CARs) with reported re-emergence in Kazakhstan where surgical incidence is about 10 out of 100,000 cases [34]. Hospital records in Kyrgyzstan show that the annual incidence of human CE cases from 1998-2000 was about 14 to 20 cases per 10,000 patients. In Tajikistan, this incidence was significantly higher than that of Kyrgyzstan, which was 27 cases per 10,000. Also, a serological survey report reveals that up till 2000, a total of 167,300 persons were found seropositive across the world for *Echinococcus* out of which 12,520 were Uzbek nationals [35]. In Afghanistan, though there has been no comprehensive study on the prevalence of CE, clinical presentations have been reported in Afghan immigrants and the US soldiers returning to the USA after duty in Afghanistan [36,37]. The prevalence results of the present study suggest butchers as one of the important occupational risk groups for CE. Many Asian and other developing countries where CE is endemic should be aware of the infection in butchers and disseminate knowledge to prevent and control the disease.

In this study, we investigated various demographic factors like age and literacy levels of butchers as factors influencing CE. Both factors were found significantly associated with seroprevalence of CE among butchers. This observation of higher seroprevalence in individuals above 30 years old is in accordance with CE prevalence observation in Heilongjiang province of China, where higher CE infection was observed in individuals between 41-45 years of age [16]. This is expected due to the slow development of hydatid cyst in intermediate hosts and humans as well as increase exposure rate as age increases. In several studies, report of significantly higher seropositivity for CE with increasing age has been documented [38,39].

Table 5. Final binary logistic regression analyses for the prediction of *Echinococcus granulosus* in abattoir personnel.

Category	Exposure variable (n)	Comparison (n)	p-value
Age	More than 30 (145)	Up to 30 (219)	0.039*
Work experience	> 10 years (209)	Up to 10 years (155)	0.011*
Animal Slaughtered	Small (94)	Large (270)	0.020*
Literacy	Up to 8 th standard (321)	More than 8 th standard (43)	0.042*
Association with dogs	Yes (228)	No (136)	0.037*
Contact with dog feces during disposal	Yes (278)	No (86)	0.035*
Awareness about eating with unwashed hands	No (154)	Yes (210)	0.041*

* significant difference ($p < 0.05$).

Furthermore, epidemiological studies have signaled out increasing age and home slaughtering practices as very important factors contributing to CE infection in China [40]. This was also corroborated by Yuan and colleagues, who found a 1.55% CE prevalence in humans from five provinces spotlighting the significant association of home slaughtering with the observed infection rate [41]. The present study agrees with the observation of Yuan and colleagues, as Pakistan, a being border-sharing neighbor of China, and also endemic for human CE is characterized by unmechanized and poor abattoir facilities with frequent home-slaughtering.

Also, years since involvement in the butchering profession was found to be a significant factor ($p < 0.05$) for seropositivity which is in contrast to the findings of Youssefi *et al.* [28]. Type of animal slaughtered was also investigated as a possible risk factor contributing to CE prevalence and results showed that higher prevalence and risk of seropositivity in the butchers slaughtering small (sheep and goat) ruminants as than the butchers slaughtering large animals (cattle and buffalo). This can be attributed to the fact that in the selected study areas, public mostly prefer mutton as compared to beef and thus the magnitude of sheep and goat slaughtering is far higher than that of cattle and buffalo. Additionally, in areas selected for this study, sheep and goats are reared on open grazing fields which are contaminated with dogs' feces as compared to buffalo and cattle which are mostly fed in confined areas.

This study demonstrates a significant correlation with the literacy level of Pakistani butchers with prevalence almost two times higher in the less educated group than the group with secondary education. Although this is in contrast to a previous questionnaire based survey, where 95.8% of participants (277/289) had no knowledge of zoonotic diseases and CE which could be attributed to poor education, yet no correlation between CE and education level was found [3]. In Iran, the influence of education level was also not significant [42]. Meanwhile, in other studies, Ozcelik *et al.* [43] and Attanasio *et al.* [44] found a significant impact of education in the control/reduction of CE incidence in Turkey and Italy, respectively. In Peru, education was demonstrated as a key determinant of people's perception of CE. Those who had seen a cyst (OR = 3.82) and those who have no background knowledge about CE (OR = 4.7) were more likely to be CE positive [45]. This disparity between educated and non-educated participants may be attributed to unequal inclusion from each category [37].

In this study, we found that knowledge of CE was very low among participants which is also true in other regions of Pakistan [3] where only 4.2% (12/289) of the participants (butchers) were aware of CE. In this survey, 29 (13.80%) seropositive butchers knew the dangers of eating food products contaminated with dogs' feces. The level of awareness among these butchers on the risk factors was lower than the observations made by Khan *et al.* [3] where 51.2% of the questioned population was aware of the consequences of eating contaminated food. We also found higher seroprevalence in butchers who had encountered hydatid cyst(s) but the difference was insignificant.

Many countries where CE is endemic also have high dog population [46] and Molan *et al.* [47] found a very high prevalence (50%) in dogs in Iraq. Keeping a shepherd or pet dog is considered to be a potential risk factor for human CE [48]. The seroprevalence reported among butchers in the present study showed a relationship with dog contact. We also found a higher prevalence however insignificant in the butchers who confirmed the presence of feral dogs in their surroundings. Our results were in coherence with a previous study in which 77.2% (142/184) of dog owners reported the presence of stray/feral dogs around their vicinity and were aware that they may contract cystic echinococcosis due to close association with dogs [3]. We also found a statistically significant association between seropositivity and contact with dogs' feces during improper disposal. Similar findings have also been documented in Morocco and Tanzania [49, 50].

CE is distributed worldwide and has endemic foci in every inhabited continent. From Pakistan, only a few reports on animals and humans are available before this investigation was carried out among butchers. Although the study encountered a few limitations including the lack of confirmatory tests. However, the ELISA kit used in this study demonstrated high sensitivity and specificity for hydatid disease according to previous studies [21,22] and is relatively inexpensive and easy to perform. Moreover, ELISA test can be used for mass-scale screening of populations. Additionally, we acknowledged the sample size and study area which involved a few prefectures as limitations to the current study. Nonetheless, we suggest that future studies consider a larger coverage (study area and sample size) encompassing different provinces to provide a better outlook on the status of CE in abattoir personnel and to devise control strategies in this occupational risk group.

Like other developing countries, Pakistan lacks the platform to execute integrated CE control strategies. There is therefore a need for the responsible government agencies to enact policies or enforce existings ones to ensure funding is readily available to the provincial and district (covering both urban and rural areas) governments in order to establish a One-Health approach to create awareness about CE and its associated risk factors among the masses. The results of this study will also prove useful in similar developing countries like Pakistan in devising control strategies, particularly among slaughterhouse workers.

Conclusions

In general, our results showed that abattoir personnel in Pakistan are exposed to CE and provide valuable information on the epidemiology of hydatidosis in Pakistan, with very significant implications in designing prevention plans. This report is not only a warning for native slaughterhouse personnel to be aware of the risk of infection but also demonstrates global significance especially in developing countries with poor abattoir infrastructure and limited CE awareness.

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Authors' Contributions

MAA, LL, HBY and WJZ designed the study. MAA, AH, MS and AAA performed the sampling. MAA, MHT, WQ, MWY and MS did the formal analysis. MAA wrote the original draft while JAO, WJZ, HBY and BQF carried out review and editing. WJZ acquired the funding.

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