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Serological evidence of Hepatitis E Virus infection in pigs and jaundice among pig handlers in Bangladesh

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Key words: Hepatitis E Virus, pigs, pork, pig handlers, jaundice, Bangladesh, Zoonotic HEV

Summary

Hepatitis E Virus (HEV) is the most common cause of viral hepatitis in humans. Pigs may act as a reservoir of HEV and pig-handlers were frequently identified with a higher prevalence of antibodies to HEV. The objectives of this study were to identify evidence of HEV infection in pigs, and compare the history of jaundice between pig handlers and people not exposed to pigs and pork.

Blood and fecal samples were collected from 100 pigs derived from three slaughterhouses in the Gazipur district of Bangladesh from January to June, 2011. We also interviewed 200 pig handlers and 250 non-exposed people who did not eat pork or handled pigs in the past 2 years. We tested the pig sera for HEV-specific antibodies using a competitive ELISA and pig fecal samples for HEV RNA using real time RT-PCR.

Out of 100 pig sera, 82% (n=82) had detectable antibody against HEV. Of the 200 pig handlers, 28% (56/200) demonstrated jaundice within the past two years, whereas only 17% (43/250) of controls had a history of jaundice ($p<0.05$). Compared to non-exposed people, those who slaughtered pigs (31% vs. 15%, $p<0.001$), reared pigs (37% vs. 20%, $p<0.001$), butchered pigs (35% vs. 19%, $p<0.001$), or were involved in pork transportation (28% vs. 13%, $p<0.001$) were more likely to be affected with jaundice in the preceding two years. In

multivariate logistic regression analysis, exposure to pigs (Odds ratio [OR]: 2.2, 95% CI: 1.2-3.9) and age (OR: 0.97, 95% CI: 0.95-0.99) was significantly associated with jaundice in the past two years.

Pigs in Bangladesh demonstrated evidence of HEV infection and a history of jaundice was significantly more frequent in pig handlers. Identifying and genotyping HEV in pigs and pig handlers may provide further evidence of the pig's role in zoonotic HEV transmission in Bangladesh.

Impacts

- Pigs may act as a reservoir of Hepatitis E Virus (HEV) and pig-handlers were frequently identified with a higher prevalence of antibodies to HEV.
- We found that 82% of pigs in Bangladesh had detectable antibody against HEV.
- Compared to non-exposed, people those who have exposure to pigs (slaughtered pigs, reared pigs, or butchered pigs), were more likely to be affected with jaundice in a two year period preceding our study.

Introduction

Hepatitis E Virus (HEV) is the most common cause of viral hepatitis globally (Labrique *et al.*, 1999). HEV has four major genotypes (1, 2, 3, and 4) and all of them infect humans. Genotypes 1 and 2 are transmissible among humans only, whereas Genotypes 3 and 4 are zoonotic in nature and prevalent both in low and high-income countries (Cooper *et al.*, 2005, Meng *et al.*, 2002). Antibodies against HEV have been found in a wide variety of domestic animals, including pigs (Zhang *et al.*, 2008). As has been demonstrated in other parts of the world, HEV is endemic among pigs in those regions surrounding Bangladesh, particularly within India, Nepal, China, and Japan (Clayson *et al.*, 1995, Takahashi *et al.*, 2003, Shukla *et al.*, 2007, Vivek & Kang, 2011, Zhang et al., 2008). However, HEV in pigs had not been previously reported in Bangladesh.

Acute HEV infection in humans is associated with clinical features that include jaundice, dark colored urine, fatigue, vomiting, and abdominal pain (Ryder & Beckingham, 2001). Jaundice is a common clinical feature for any viral hepatitis and is considered as an important clinical manifestation and the most probable indicator of HEV infection (Gupta *et al.*, 2011). More than one third of hospitalized jaundice patients are associated with underlying HEV infection (Gupta et al., 2011). Animal handlers, such as farmers, have been shown to be at increased risk of HEV infection (Lee *et al.*, 2013). A recent study conducted in India found 94% of pig handlers had antibodies against HEV, a rate higher than rural (59%) and urban (73%) controls (Vivek & Kang, 2011). Similar findings were reported from China, Thailand and the USA (Meng, 2009, Meng, 2010, Cooper et al., 2005).

Although predominantly a Muslim country, in Bangladesh there is a significant number of Christian households that rear pigs for personal consumption (Nahar *et al.*, 2013). The pig population in Bangladesh is estimated to be approximately eight million (Khan *et al.*, 2014). There are some regions where pig rearing is popular, including the districts of Rajshahi, Chapai Nawabganj, Mymensingh, Noagan, Gazipur and Tangail. Moreover, there is a government pig farm in Rangamati (Islam *et al.*, 2006, Khan et al., 2014, Nahar et al., 2013). HEV has been a leading cause of hepatitis in Bangladesh and testing whether zoonotic transmission of HEV occurs in Bangladesh is important in this regard. We conducted this study to identify evidence of HEV infection in pigs and to compare the history of jaundice between pig handlers and people not exposed to pigs and pork.

Methods

Pig HEV serology

From January to June 2011, we collected blood and fecal samples from pigs slaughtered in three abattoirs within the Gazipur district of Bangladesh. We recorded demographic information including age (in months), sex, breed (indigenous vs crossbred), and herd size of the pigs (in number). Blood samples were kept at room temperature for 30 minutes and serum was separated by centrifugation and preserved at 2-8°C in a cold box before being transferred to icddr,b's animal laboratory where they were stored at -20°C until testing.

Anti HEV serology and testing for HEV RNA

Anti-HEV antibodies were detected by commercial HEV ELISA kit (MP Biomedicals, Singapore) according to the manufacturer's protocol (Kaufmann *et al.*, 2011). Initially, reactive samples were re-tested and those with repeated reactive results were considered positive. According to the manufacturer, the kit possesses 97% sensitivity and 98% specificity for the detection of HEV antibodies. The kit is based on a double-antigen sandwich ELISA, which permits detection of all groups of immunoglobulin (IgG, IgA, IgM) in all animal species (Andraud *et al.*, 2014). We then tested the sera and fecal samples of all 100 pigs by real time reverse transcriptase polymerase chain reaction (RT-PCR) as described previously (Gyarmati *et al.*, 2007).

Human jaundice and exposure to pigs

We enrolled people working with pigs or pork-processing from the regions primarily known for pig rearing in Bangladesh (Rajshahi, Chapai Nawabganj) (Khan et al., 2014), as well as a pig slaughter house in Gazipur (Haider *et al.*, 2012). We defined a person as a pig or pork handler who had been involved with any one of the following activities for the past two years: rearing pigs, slaughtering pigs, butchering pigs, selling pork, transporting pork or eating pork. We then enrolled non-pig or pork-exposed control individuals from the same geographical regions and confirmed that they were not involved in any of the activities mentioned above within the past two years.

We defined persons as jaundiced when they reported yellowish discoloration of the sclera as well as dark or yellow colored urine, or any person having laboratory diagnosis of increased bilirubin levels (>3mg/dl) within the past two years (Gupta et al., 2011). All enrolled individuals were interviewed regarding demographic information, exposure to pigs and pork, and a history of jaundice through a structured questionnaire.

Sample size estimation:

We estimated the sample size necessary to detect a significant difference between exposed and non-exposed human subjects enrolled in this study. Considering a hypothetical proportion of 20% jaundiced people in the pig exposed group and 10% jaundiced people in the non-exposed group, with a 95% confidence interval and 80% power, our estimated sample size was 286 for each group. We were able to enroll 250 people in non-exposed groups, 200 people in the exposed group due to lack of resources and time constraints and, most importantly, somewhat fewer pig raisers within a predominantly Muslim country. For studying HEV sero-prevalence in pigs, we considered a 60% seroprevalence of HEV antibodies in pigs based on the published literature of neighboring countries (Clayson et al., 1995, Shukla et al., 2007, Zhang et al., 2008) and estimated that 270 pigs should be enrolled (AusVet: Animal Health Services, 2016) . However, we were only able to enroll 100 pigs because of limited resources as well as the constraints listed above.

Data analysis

We stratified the sero-prevalence of HEV in pigs by age, sex, breed, and herd size. We classified the human exposure group into two primary groups: exposure to pigs and exposure to pork. The people who were primarily involved with rearing pigs, slaughtering pigs and butchering pigs were grouped as “exposure to pigs”. In contrast, those who exclusively ate pork, worked as salesmen, or who were involved in transporting pork were classified as "exposure to pork". We used the chi-square or Fisher's exact test to identify the association between people having jaundice with their exposure to pigs (rearing pigs, slaughtering pigs and butchering pigs) or pork (eating pork, working as salesmen, and transporting pork). We performed univariate and multivariate logistic regression analysis to identify significant risk factors for acquiring jaundice in the past two years.

Ethical approval:

The study was part of a study at American International University of Bangladesh and the study protocol was approved by the ethical committee of the university. All participants and pig owners provided oral consent before they were enrolled in the study.

Results:

Serum samples were collected from 100 pigs in three slaughterhouses located within the villages of Kaligonj sub-district of Gazipur district of Bangladesh. Although the slaughterhouse owners collected pigs from different districts of Bangladesh, the majority of pigs were brought from Barisal (33%), Jessore (25%), and Gazipur districts (14%). Of the 100 pigs tested for HEV-specific antibodies, 82% (82/100) had anti-HEV antibodies.

Pig demography and HEV status

Compared to the pigs that lacked HEV antibody, pigs with HEV antibody were older [21.5 months vs. 9.6 months, $p<0.001$], were more likely to be raised in larger herds (mean herd size: 194 pigs, vs. 125 pigs, $p=0.008$), were predominantly male (60%, vs. 22% $p=0.004$), and were more likely to be an indigenous breed (89% vs. 39%, $p<0.001$). No HEV RNA was detected from any fecal samples.

Exposure to pigs and Jaundice history of pig handlers

We enrolled 450 persons from 12 districts of Bangladesh: 200 were pig handlers and 250 were controls who never handled pigs or eat pork. Among 450 persons, 46% (n=206) were Christian, 40% (n=181) were Muslim, and the rest (14%)(n=63) were Hindu. Of the

200 pig handlers, 28% (n=56) demonstrated jaundice within the past two years, whereas only 17% (n=43) of controls had a history of jaundice ($p<0.05$). The mean age of people having jaundice was 33.2 years compared to 37.6 years for controls ($p<0.05$).

People had different levels of exposure to pig and pork: 24% (n=107) were involved in rearing pigs, 18% (n=82) worked at a slaughter house, 23%(103) were involved in butchering pigs, 17% (n=77) were involved in transportation of pigs, 16% (n=16) were involved in selling pork, and 50% (n=226) had eaten pork within the past two years. The people who were exposed to pigs (OR: 2.2, 95% CI: 1.2-3.9) were more likely to be affected with jaundice whereas older aged people demonstrated a low risk of having jaundice (OR: 0.97, 95% CI: 0.95- 0.99) compared to younger adults.

Discussion

This study demonstrated that pigs in Bangladesh have been exposed to the Hepatitis E Virus. Anti-HEV antibodies have been demonstrated among pigs in several HEV-endemic and non-endemic countries, including India, China, Nepal, Taiwan, USA and Canada (Vivek & Kang, 2011, Zhang et al., 2008, Zhang *et al.*, 2009, Zhuang, 1991, Clayson et al., 1995, Wu *et al.*, 2002, Meng et al., 2002, Dalton *et al.*, 2008, Yoo *et al.*, 2001). The prevalence of anti-HEV antibodies among pigs in this study was similar to other endemic countries in this region including China (83%) (Zhang et al., 2008), Nepal (85%) (Clayson et al., 1995) and India (94%) (Shukla et al., 2007). The pigs enrolled in the study were reared locally within the country, indicating that these pigs were probably exposed to a locally circulating HEV virus.

We found a variation of anti-HEV antibody status of pigs according to age, sex, breed and herd size. The differences in ages may be indicative to a cumulative life-time exposure to an as yet an unknown environmental source which is consistent with other reports (Meng, 1997). Pigs sampled from larger herds had HEV antibody more frequently than the smaller herds could be due to cumulative exposure to larger number of animals, and is consistent with other studies (Yoo et al., 2001). The differences in HEV antibodies in breed might be associated with the differences in rearing systems as well as herd size because cross bred pigs are reared mostly in small scale farming systems and indigenous breeds are primarily reared in backyard farms (Khan et al., 2014). The reasons for the differences in the breed as well as sex should be explored in future studies.

Our study findings demonstrated that people exposed to pigs had a significantly higher risk of jaundice in Bangladesh. A recent study showed that 19 to 25% of all maternal deaths and 7 to 13% of all neonatal deaths in Bangladesh were associated with jaundice in pregnant women (Gurley *et al.*, 2012). In previous studies, 58% of deaths in pregnant women with ongoing acute liver disease were associated with HEV infection (Gurley et al., 2012). In Bangladesh, 30–60% of the acute viral hepatitis patient has an underlying HEV etiology (Labrique *et al.*, 2009). Recently, a number of human HEV outbreaks in Bangladesh were associated with contaminated drinking water (Haque *et al.*, 2015). However, there is little information available regarding the zoonotic transmission of HEV in human cases in Bangladesh.

Exposure to pigs and resultant jaundice makes biological sense because HEV is known to be transmitted by a fecal-oral route within animals species, from animals to humans in infectious body fluids, and from contaminated food or water sources to humans and other animals (Meng, 2009). Insufficient disposal and poor management of sewage and contamination of drinking and irrigation water was responsible for many HEV epidemics in developing countries (Fu *et al.*, 2010). Thus people working in some activities with pigs would have greater risks of viral exposure. In contrast, people who are simply exposed to pork should have a minimal chance of live viral infection because the cultural norm in Bangladesh is to cook meat above boiling temperature sufficient to kill HEV (Barnaud *et al.*, 2012). The fact that older people were seemingly protected could be due to their cumulative viral exposure to various agents of hepatitis, including HEV, which is supported by earlier studies (Dalton *et al.*, 2011).

Although we did not detect HEV RNA in our samples, it should be noted that detection of HEV from pigs depends on several factors, including the age of the pigs. Previous studies have shown that pigs only shed virus between the ages of 2 to 4 months (Cooper et al., 2005) and infected piglets generally have only a transient viremia lasting for 1 to 2 weeks shedding virus in feces for about 3 to 7 weeks (Meng, 2009). Likewise, the serum samples we tested were collected only from adult pigs which would explain why we did not detect any HEV RNA. Further exploration into the circulating genotype of HEV in pigs in Bangladesh is important, as it could help target specific interventions for human HEV infection from zoonotic and/or other environmental sources.

Limitations:

We classified people as jaundice based on clinical history and laboratory results. In fact, most of the enrolled patients could not show the laboratory results and therefore we had to rely on clinical history. Clinical signs and their history are highly subjective and typically embraces recall bias. Therefore, there is a chance of misclassification of the people classified as jaundice. Although we could not rule this out, further studies with laboratory confirmation will allow us better understanding the potential role of zoonotic HEV among jaundice or hepatitis patients in Bangladesh. Too, there is potential risk that people in the non-pig exposed group are different by a number of characteristics compared with those exposed to pigs. The difference could result from other population level differences rather than exposure to pigs and pork. These might include specific diets, which could be heavily influenced by religion and culture, and water sources that are strongly influenced by income. However, detection of differences in occurrences of jaundice makes biological sense and worth confirmation through future studies.

Conclusion:

This study provides evidence that pigs in Bangladesh are exposed to HEV. Presence of anti-HEV antibodies in pigs has important implications for public health in Bangladesh since people exposed to pigs had a significantly higher risk of jaundice. Identifying HEV genotypes in pigs could shed light on the extent of zoonotic HEV transmission in Bangladesh and provide a platform for public health intervention strategies.

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Tables:

Table-1: Status of Hepatitis E Virus antibodies among pigs slaughtered in three slaughterhouses in Kaligong sub-district of Gazipur district of Bangladesh, January –June, 2011.

Table-2: Univariate and multivariate logistic regression analysis on the factors associated with jaundice of pig handlers and non-exposed people enrolled from different districts of Bangladesh between January-June, 2011.

Table-1: Status of Hepatitis E Virus antibodies among pigs slaughtered in three slaughterhouses in Kaligong sub-district of Gazipur district of Bangladesh, January –June, 2011.

	HEV antibody positive pigs N=82 n (%)	**HEV antibody negative pigs N=18 n (%)**	**P value**
Sex of the pigs - male (vs. female)	49 (60)	4 (22)	0.004
Breed of the pigs – indigenous (vs. cross breed)	73 (89)	7 (38)	<0.001
Mean number of pigs in herd	193	125	<0.001
Mean age of pigs (in months)	21	10	<0.001

Table-2: Univariate and multivariate logistic regression analysis on the factors associated with jaundice of pig handlers and non-exposed people enrolled from different districts of Bangladesh between January-June, 2011.

		Univariate analysis			**Multivariate analysis**
		Jaundice (N=99) n (%)	**No Jaundice (N=351) n(%)**	**P value**	**Adjusted odds ratio (95% CI)**
Exposure to pigs	1. Slaughtering pigs	31 (31)	51 (15)	<0.001	2.2(1.2-3.9)
	2. Butchering pigs	35 (35)	68 (19)	<0.001	
	3. Rearing pigs	37 (37)	70 (20)	<0.001	
Exposure to pork	4. Transporting pigs or pork	28 (28)	49 (13)	<0.001	0.95(0.5-1.7)
	5. Selling pork	19 (19)	54 (15)	0.37	
	6. Eating pork	57 (57)	177 (48)	0.11	
Mean age of the participant	(In years)	33.2	37.6	P =0.003	0.97(0.95-0.99)

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