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BRIEF REVIEW

Occurrence and investigation of enteric viral infections in pigs with diarrhea in China

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Abstract Between February 2011 and February 2012, 985 and 324 samples were collected from diarrheal and healthy pigs, respectively, to detect porcine epidemic diarrhea virus (PEDV), porcine kobuvirus (PKoV), porcine bocavirus (PBoV), porcine group A rotavirus (GARV), and transmissible gastroenteritis virus (TGEV). PEDV and PKoV clearly predominated in diarrheal pigs. Compared to healthy pigs, a substantial prevalence of mixed infections was observed in diarrheal pigs (72.3 %) ($P < 0.001$). All of the coinfections were grouped into 13 patterns. The results of quantitative PCR showed PEDV in diarrheal pigs had a slightly higher mean viral load than that in healthy pigs (7.9×10^6 versus 2.0×10^5 copies/g of stool), while similar mean viral loads were observed for PKoV and PBoV. These findings reveal the severity of coinfections in diarrheal disease and suggest that attention should be paid to synthetic administration and vaccination for its prevention and control.

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Diarrhea is a common disease in pigs, and the associated dehydration is a leading cause of death, which causes considerable economic losses in the pig industry. The etiological agents of diarrhea are varied, including multiple viral, bacterial, and parasitic pathogens, but viruses are the predominant factor [8, 16, 23]. The most common causal agents are porcine epidemic diarrhea virus (PEDV), transmissible gastroenteritis virus (TGEV), porcine group A rotavirus (GARV), and emerging viruses, including porcine kobuvirus (PKoV) and porcine bocavirus (PBoV) [5, 14, 20, 25]. Recently, PEDV has been of great concern in Asia, especially in China, Korea, Vietnam, and Thailand [4, 7, 18, 19]. It was reported that 50.4 % of 1,258 enteric cases were attributable to PEDV, and the mortality approached 100 % [3, 19]. PKoV is a new possible etiological agent belonging to the genus *Kobuvirus*, family *Picornaviridae* [20], which has been found frequently in both healthy and diarrheal pigs in recent years [1, 17]. Park et al. reported that the incidence of PKoV in diarrheal pigs was as high as 84.5 % and presumed it was associated with diarrhea. GARV was also frequently detected in diarrheal pigs [17]. Furthermore, TGEV is a highly contagious enteric disease of piglets, which has been reported in most countries worldwide. Many studies have also indicated that PBoV, which has close relationships with enteric infections in animals, especially in younger animals, had a high prevalence in stool samples of pigs [5].

The investigation and diagnosis of etiology is of great significance for protection from infectious diarrheal diseases. Many countries throughout the world have paid considerable attention to etiology [16, 23]. However, most studies have concentrated on a single pathogen, and limited data are available regarding mixed infections [5, 12]. Thus, we conducted a systematic investigation, which covered epidemiology, pathology, and etiology. We described the

clinical signs and pathological findings in infected pigs during diarrhea outbreaks and determined the distribution of single and multiple infections with these viral agents during diarrhea. Our third objective was to compare the prevalence of enteric viruses in pigs with diarrhea with that in healthy pigs.

Between February 2011 and February 2012, a total of 985 clinical fecal and intestinal samples were collected from pigs with diarrhea of different ages on 143 farms in 11 Chinese provinces. We also processed 324 fecal samples of clinically healthy pigs from the same locations as pigs with diarrhea. Fresh fecal samples were collected from individual pigs on farms, placed in sterile specimen cups, stored in nitrogen canisters or kept on ice, and returned to the laboratory within 12 h. Intestinal samples containing yellow fluidic contents were obtained during postmortem inspections at diagnostic laboratories. All samples were processed immediately or stored frozen (−20 °C).

Histopathological analysis was performed for cases with representative diarrheal symptoms. To detect specific antigens in the infected tissues, immunohistochemistry was also performed to study a series of formalin-fixed paraffin-embedded samples. The primary antibodies (targeting the TGEV-S and GARV-VP6 protein, respectively) were kindly provided by Dr. Qigai He of our team, and mouse anti-S monoclonal antibody was purchased from Korea (Catno: 9191, JBT, Korea). A Histostain-Plus Kit (Zymed Laboratories, South San Francisco, United States) was used for detection of these viral antigens.

Viral RNA and DNA were extracted using a QIAamp Viral RNA and DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. Viral cDNA synthesis was performed using random hexamer primers (TaKaRa Bio Inc., Otsu, Japan) and the cDNA was used immediately for amplification or stored at –80 °C.

The base sequences and the predicted sizes of the amplified products for the specific primers used in this study are shown in Supplementary Table 1 [21, 25]. All of the primers were synthesized commercially (with finishing performed by Sangon Biological Engineering Technology Inc.). The appropriate positive controls were generated from cloned gene fragments by PCR using a GeneAmp PCR system 9700 instrument (Applied Biosystems, Foster City, CA). The controls yielded the expected bands and were confirmed subsequently by sequencing. All tests were repeated in duplicate in parallel with relevant positive and negative controls. Discrepant results were investigated further, and the samples were sequenced.

Quantitative PCR was also performed. In order to create a standard curve, recombinant plasmids containing the target genes were constructed using the primers for quantitative PCR (Supplementary Table 1) [11, 13]. Amplifications were performed with Premix Ex Taq^{TM} (Probe qPCR) (TaKaRa Bio Inc., Otsu, Japan) in an ABI StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), and the quantitation of the viral load in positive samples was done on the basis of their cycle threshold (Ct) values by employing the standard curve of Ct values plotted against known concentrations of tenfold serially diluted standard plasmids.

Clinical data were collected by retrospective analysis of the protocols. Chi-square or Fischer's exact tests were performed as necessary using SPSS version 12.0 (Chicago, IL, USA). Results were considered statistically significant for two-tailed P-values of <0.05.

During the study period, clinical data on diarrheal cases were systematically collected via verbal enquiries and questionnaires that were sent to veterinarians or technical directors of farms. The diarrheal disease initially involved sporadic outbreaks among fattening pigs, with an incidence of 5–10 % on most pig farms. Infected pigs mainly presented a black spray-like stool, which had a characteristic fetid smell. Fortunately, herds recovered after 1 week. Infected nursery pigs had similar symptoms to fattening pigs and they were cured by intravenous rehydration and antipyresis. Approximately 15–20 % of pregnant sows was infected progressively, and they were characterized by black watery diarrhea and anorexia. Vomiting also affected a few pigs. These symptoms usually lasted 1–3 days, but no mortality was observed.

Compared with other herds, piglets were more susceptible within 1 week of farrowing. The diarrheal diseases spread rapidly and caused outbreaks throughout barns within 3–5 days. Obvious clinical signs were yellow watery stool, vomiting, depression, and anorexia, while death from dehydration often occurred after 2–3 days of sickness. Necropsies of deceased piglets detected a mass of curdled and undigested milk in their stomachs. The small intestine wall was thin and almost transparent. Overall, no effective measures could be found to cure the piglets if they were infected within 1–7 days of birth, and the mortality rate reached 80–100 %. The mortality rate of 1- to 2-week-old piglets could be reduced to 20–30 % by keeping them warm, disinfecting the environment, and providing oral rehydration salts and antipyretics. If the piglets were infected >14 days after birth, the mortality rate was low.

The histopathological analysis showed that pathological changes were mainly detected in the jejunum and ileum, with little change in the duodenum. Typical observations were microscopic lesions or villus atrophy with the replacement of columnar villus epithelial cells by flat to cuboidal cells (Fig. 1A2). Some enterocytes showed marked cytoplasmic vacuolation (Fig. 1A3). In some cases, high levels of villous atrophy were accompanied by considerable shortening and thickening of the villi, which even led to the disappearance of the lamina propria.

Immunohistochemical staining results of infected tissues are shown in Fig. 1. By using the specific anti-PEDV and anti-GARV monoclonal antibodies, intense immunostaining for PEDV and GARV was observed in the cytoplasm of epithelial cells, indicating the presence of viral antigens. In some cases, both PEDV and GARV could be detected. This supported the idea that pathogenicity in combined infections could be associated with a combination of multiple pathogens.

As has also been shown by Martelli et al. in Italy, Khamrin et al. in Thailand, and Park et al. in Korea [9, 15, 17], our study shows that the pathogens causing diarrhea are diverse. Of the 985 diarrheal samples, 96.2 % cases had at least one viral pathogen, whereas no virus could be detected in the remaining 37 samples. PEDV clearly predominated ($P < 0.001$), with 807 cases (81.9 %; 137 in single infections and 670 in mixed infections). The detection rate agreed well with a report by Sun et al. in which 82.0 % of fecal samples were found to be positive for PEDV [24]. However, the incidence of PEDV was much lower in Korea (50.4 %) and the Czech Republic (18.8 %) [3, 22]. Like previously published reports from Brazilian and Thailand [1, 9], we also confirmed that PKoV was frequently detected in diarrheal samples (75.3 %; 89 in single infections and 653 in mixed infections). Notably, Aichi virus, which is a kobuvirus, has been isolated successfully and shown to cause diarrhea in humans [28], but PKoV has not yet been successfully cultured. Therefore, it is necessary to pay close attention to the isolation of PKoV and explore its pathogenic mechanism, which will clarify the risk and pathogenicity of this virus to pigs and other hosts. PBoV and GARV infection were identified in 307 (31.2 %) and 95 (9.6 %) samples, with six and four single infections, respectively. TGEV was the least frequently detected, and it was found only in six mixed cases. Compared with PKoV and PBoV, PEDV had a slightly higher mean viral load (1 log unit higher) in diarrheal pigs (Fig. 2) (7.9×10^6 versus 3.1×10^5 and 4.6×10^5 copies/g of stool, respectively).

Coinfections with multiple pathogens make it difficult to control infectious diseases, and they are receiving increased attention from researchers [17, 26]. Unfortunately, limited data are available on coinfections during gastroenteritis in pigs. In this study, we observed a high percentage of mixed infections (75.1 %) among clinically positive samples. Dual, triple, and quadruple infections accounted for 43.9 %, 26.1 %, and 2.3 % of cases, respectively. All of the coinfections were grouped into 13 patterns, and the number of cases showing each pattern is listed in Table 1. PEDV + PKoV was clearly the most frequent viral coinfection pattern (35.9 %, 354 out of 985) ($P < 0.001$). However, this disagrees with a previous report that showed that PKoV + GARV was the most common coinfection pattern in Korea [17]. According to this survey, single infection with PEDV occurred in only 13.9 % of the cases, while most of cases involved mixed infections. In this regard, although on the whole, PEDV was the most frequent viral agent detected in diarrheal samples ($P < 0.001$), it should be considered that various viruses may co-exist with PEDV. It is well established that acceleration of disease is often seen during mixed infections

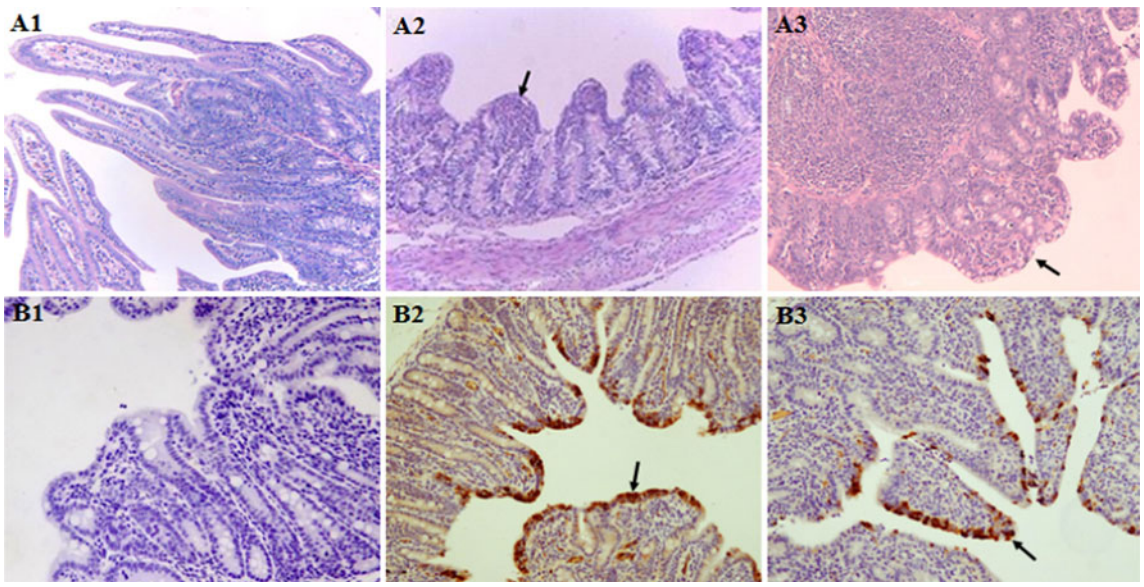

Fig. 1 Intestinal histology (**A1**, **A2**, **A3**; magnification, ×100) and immunohistochemical analysis (**B1**, **B2**, **B3**; magnification, ×200) of infected pigs. (**A1**) Control; (**A2**) lesion of the jejunum; (**A3**) lesion of the ileum; (**B1**) control; (**B2**) PEDV-infected tissue; (**B3**) GARV-infected tissue

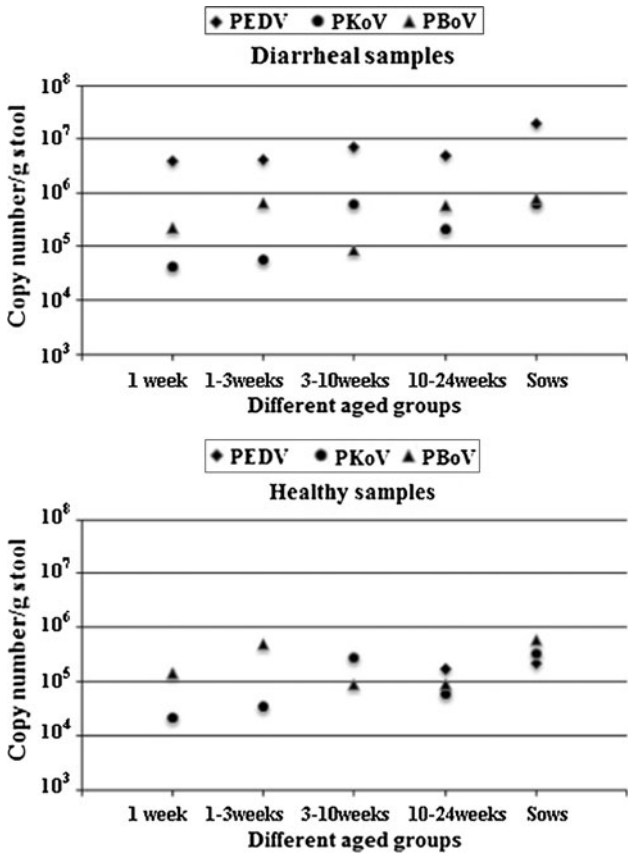

Fig. 2 Mean viral loads of different age groups for PEDV, PKoV and PBoV in diarrheal and healthy pigs

because of the compound nature of the viral cytopathic effects affecting the host in a negative manner [6].

We also analyzed the etiology of disease in relation to the age of the infected pigs. PEDV was more common in suckling (89.1 %) and newborn pigs (87.6 %) ($P < 0.001$). The prevalence of PKoV was not much different between different age groups. PBoV was more prevalent in grower pigs (52.6 %), while GARV was more prevalent in suckling (15.6 %) and nursery (22.2 %) pigs. Moreover, different infection patterns were observed in different age groups (Table 1). Single infections were significantly more common in nursery pigs ($P < 0.001$), and in this group, PKoV single infections were identified most frequently (24.2 %). It is noted that mixed infections were found mainly in piglets less than 21 days old and in sows ($P < 0.001$). The former could be a result of the poorly developed immune systems of young pigs and the low protective efficacy of the colostrum and milk [2]. However, many farms take measures to prevent exposure of pregnant sows to piglet feces and gut contents half a month before giving birth, since infection inevitably leads to virus shedding and transmission.

To provide a better understanding of the role of enteric viruses during acute diarrhea, we compared the differences in virus load and distribution of enteric viruses between diarrheal and healthy cases. The samples from healthy pigs were collected from cohorts in the same locations as the diseased pigs to rule out differences due to different breeding conditions and immunization procedures. PEDV were identified in 35 healthy samples. Compared to diarrheal samples, the detection rate were lower ($P < 0.001$). Remarkably, the majority of PEDV-positive samples (77.1 %, 27 out of 35) in healthy pigs came from pregnant sows. As reported previously [24], PEDV can be disseminated by vertical transmission, so the presence of PEDV in pregnant sows would undoubtedly have contributed to the high morbidity rate in young piglets. Importantly, we found that the copy numbers of PEDV in pigs with diarrhea tended to be higher than in healthy pigs (7.9×10^6 versus 2.0×10^5 copies/g of stool). This implies that the pathogenicity of PEDV may be related to the viral load, but this relationship has not yet been established. This study showed that the incidence of PKoV in healthy pigs was 37.7 % (122 out of 324), which agreed with previous reports from China (30.1 %) and Japan (45.5 %) [10, 27]. However, healthy pigs had a mean viral load similar to that of diarrheal pigs (1.5×10^5 versus 3.1×10^5 copies/g of stool), which may account for the increased number of infections via a "silent" transmission route. PBoV was also found in 84 (25.9 %) healthy samples, and there was no significant difference in virus load between diarrheal and healthy samples (4.6×10^5 versus 2.9×10^5 copies/g of stool). TGEV and GARV were not detected in healthy pigs. Mixed infections were less common in healthy pigs, while single infections predominated.

In the outbreaks, there was other clinical picture in which many farms applied booster immunizations 2–3 times during the autumn and winter using a commercial vaccine (inactivated TGEV H and PEDV CV777), but piglets were still not protected. This may be because the PEDV field strains prevalent in China were genetically different from the CV777 vaccine strain or it may be related to the low immunogenicity of the vaccine itself [24]. PKoV may also be an important causative agent, but there is no effective vaccine to combat this virus. Other factors may have contributed to the prevalence of the epidemic, such as prolonged cold and wet weather in China, unsound cultural concepts, low scale, and nonstandard operations. The issue of immunosuppression must also be taken into account. During our investigation, the detection rate of PCV2, PRRSV, and HCV in feces continued to increase while PEDV and PKoV were epidemic (data not shown). Overall, the clinical diarrheal diseases were extremely complex and worthy of much more consideration.

In conclusion, this is the first report of an extended study of the etiology of infectious diarrheal disease conducted systematically in China. Our findings provide evidence that

Table 1 Etiology in relation to the age of diarrheal and healthy pigs

Pathogens	Number of diarrheal pigs testing positive, no. (%)					Total (n=985)	Number of healthy pigs (%) testing positive, no. (%)					
	1 week (n=581)	1-3 weeks (n=128)	3-10 weeks (n=99)	10-24 weeks (n=76)	sows (n=101)		1 week (n=115)	1-3 weeks (n=52)	3-10 weeks (n=47)	10-24 weeks (n=52)	sows (n=58)	Total (n=324)
Single infections[a]												
PEDV	83 (14.3)	23 (18.0)	14 (14.1)	6 (7.9)	11 (10.9)	137 (13.9)[c]	0 (0.0)	0 (0.0)	0 (0.0)	6 (11.5)	18 (31.0)[d]	24 (7.4)
PKoV	41 (7.1)	10 (7.8)	24 (24.2)[d]	6 (7.9)	8 (7.9)	89 (9.0)	23 (0.2)[(c)]	15 (28.9)	24 (51.1)	19 (36.5)	12 (20.7)	93 (28.7)[d]
PBoV	2 (0.3)	0 (0.0)	0 (0.0)	2 (2.6)[b]	2 (2.0)[b]	6 (0.6)	17 (14.8)	7 (13.5)	14 (29.8)[b]	12 (23.1)	5 (8.6)	55 (17.0)[d]
GAR	2 (0.3)	0 (0.0)	2 (2.0)	0 (0.0)	0 (0.0)	4 (0.4)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Mixed infections												
PEDV+PKoV	233 (40.1)[d]	53 (41.4)[d]	18 (18.2)	8 (10.5)	42 (41.6)[d]	354 (35.9)[d]	0 (0.0)	0 (0.0)	0 (0.0)	2 (3.9)[b]	3 (5.2)[b]	5 (1.5)
PEDV+PBoV	9 (1.5)	2 (1.6)	10 (10.1)[d]	10 (13.2)[d]	4 (4.0)	35 (3.6)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	5 (8.6)[d]	5(1.5)
PEDV+ GARV	10 (1.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	10 (1.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
PEDV+TGEV	2 (0.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (0.2)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
PKoV+ PBoV	5 (0.9)	0 (0.0)	0 (0.0)	6 (7.9)[d]	0 (0.0)	11 (1.1)	5 (4.3)	6 (11.5)	4 (8.5)	5 (9.6)	3 (5.2)	23 (7.1)[d]
PKoV+ GARV	12 (2.1)	2 (1.6)	4 (4.0)	0 (0.0)	0 (0.0)	18 (1.8)[b]	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
PBoV+GARV	0 (0.0)	0 (0.0)	0 (0.0)	2 (2.6)[d]	0 (0.0)	2 (0.2)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
PEDV+PKoV+PBoV	145 (25.0)	20 (15.6)	8 (8.1)[(d)]	20 (26.3)	22 (21.8)	215 (21.8)[d]	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.7)	1 (0.3)
PEDV+PKoV+GARV	11 (1.9)	8 (6.3)[c]	5 (5.1)	0 (0.0)	0 (0.0)	24 (2.4)[c]	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
PEDV+PBoV+GARV	2 (0.3)	1 (0.8)	0 (0.0)	0 (0.0)	0 (0.0)	3 (0.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
PKoV+ GARV+ PBoV	2 (0.3)	2 (1.6)	5 (5.1)	2 (2.6)	0 (0.0)[(d)]	11 (1.1)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
PEDV+PBoV+TGEV	4 (0.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	4 (0.4)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
PEDV+PKoV+PBoV+ GARV	10 (1.7)	7 (5.5)[c]	6 (6.1)	0 (0.0)	0 (0.0)	23 (2.3)[c]	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
No virus detected	8 (1.4)	0 (0.0)	3 (3.0)	14 (18.4)	12 (11.9)	37 (3.7)	70 (60.9)	24 (46.2)	5 (10.6)	8 (15.4)	11 (19.0)	118 (36.4)[d]

Statistical analysis was performed using SPSS version 12.0 (Chicago, IL, USA). *P* indicates group vs. all other age groups of diarrheal and healthy pigs or the diarrheal group as a whole vs. healthy groups as a whole. Exponents with parentheses indicate a negative association

[a] No TGEV single infections were detected

[b] $P < 0.05$; [c] $P < 0.01$; [d] $P < 0.001$

the issue of coinfections is rather serious and suggest that veterinary professionals should be aware of the threat from coinfections with multiple pathogens in China, while attention should be paid to synthetic administration and vaccination.

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