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Temporal genetic variability and host sources of *Escherichia coli* associated with fecal pollution from domesticated animals in the shellfish culture environment of Xiangshan Bay, East China Sea

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ABSTRACT

This study was conducted to analyze the genetic variability of *Escherichia coli* from domesticated animal wastes for microbial source tracking (MST) application in fecal contaminated shellfish growing waters of Xiangshan Bay, East China Sea. $(GTG)_5$ primer was used to generate 1363 fingerprints from *E. coli* isolated from feces of known 9 domesticated animal sources around this shellfish culture area. Jackknife analysis of the complete $(GTG)_5$-PCR DNA fingerprint library indicated that isolates were assigned to the correct source groups with an 84.28% average rate of correct classification. Based on one-year source tracking data, the dominant sources of *E. coli* were swine, chickens, ducks and cows in this water area. Moreover, annual and spatial changes of *E. coli* concentrations and host sources may affect the level and distribution of zoonotic pathogen species in waters. Our findings will further contribute to preventing fecal pollution in aquatic environments and quality control of shellfish.

1. Introduction

Molluscan shellfish are a valuable commodity both as a food source and as the sustaining product of an important coastal industry. Nowadays, Chinese shellfish aquaculture accounts for nearly 60% of the value of the worldwide commercial shellfish farming (Li et al., 2009). Xiangshan Bay, located at the coastline of East China Sea, is one of the biggest areas for shellfish harvest in the southeast of China. While the quality of shellfish is dependent upon many factors, clean growing water is undeniably one of the most important (Chigbu et al., 2005). Unfortunately, fecal contamination of water from agricultural wastes has become a crucial and widespread problem in the developing countries (Gu et al., 2008; Jenkins et al., 2009; Mazari-Hiriart et al., 2008; Yajima and Kootatep, 2010). Contamination of shellfish growing water by animal fecal wastes has been considered to be a vehicle for transmission of the important zoonotic pathogens (such as *Escherichia coli* O157:H7, *Salmonella* spp., *Campylobacter* spp. and *Listeria monocytogenes*) responsible for most cases of gastroenteritis illness (Abdelzaher et al., 2010; Scott et al., 2002). These fecal wastes enter water bodies by direct discharge, through surface run-off and/or seepage. However, managing and reducing fecal pollution levels in water are challenging because there are many host sources that are difficult to identify.

E. coli has been widely used as an indicator of fecal contamination since it is part of the common intestinal flora of endothermic animals and is easy to cultivate (Scott et al., 2002). There are a number of methods available for the enumeration of *E. coli* from waters; however, the presence of *E. coli* in water does not definitely indicate the source of fecal pollution. Consequently, a recently developed tool termed as Microbial Source Tracking (MST) which is defined as a variety of phenotypic and genotypic methods have been developed to distinguish fecal *E. coli* strains of different animal sources (Scott et al., 2002; Stoeckel and Harwood, 2007). The phenotypic methods include antibiotic resistance profiles (Carroll et al., 2009), carbon utilization profiles (Hagedorn et al., 2003), and whole-cell fatty acids (Duran et al., 2009). Due to the unstable phenotypes, low sensitivity at the intraspecies level and limited specificity, the practical feasibility of phenotypic methods are controversial. The genotypic methods include the fingerprints generated from ribotyping (Scott et al., 2003), pulsed-field gel

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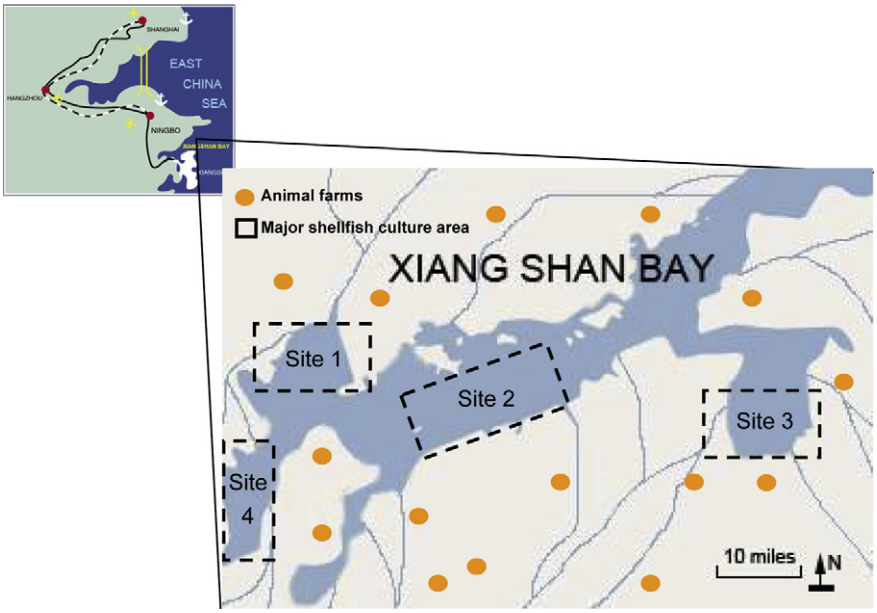

Fig. 1. Map of Xiangshan Bay showing the distribution of a number of farms around the shellfish aquaculture areas and water sampling sites 1 (oysters culture area), 2 (clams culture area), 3 (mussels culture area) and 4 (scallops culture area).

electrophoresis (PFGE) (Nayak and Stewart-King, 2008), and repetitive extragenic palindromic (rep)-PCR (Lyautey et al., 2010). Most of these MST methods are based on the hypothesis that phenotypic and genotypic traits of specific strains are associated with a specific animal. On the basis of this hypothesis, a fingerprint (phenotypic or genotypic profiles) library of strains from known sources has been developed to predict the source(s) of unknown water isolates.

Rep-PCR, which can differentiate between closely related strains of bacteria, and can be used for high-throughput studies, is considered as one of the most effective MST methods (Dombek et al., 2000; Johnson et al., 2004). In rep-PCR DNA fingerprinting, PCR amplification of the DNA between adjacent repetitive extragenic elements is used to obtain strain-specific DNA fingerprints which can be easily analyzed with pattern recognition computer software. Four methods, namely REP-PCR, ERIC-PCR, BOX-PCR and $(GTG)_5$-PCR, are frequently used in rep-PCR fingerprinting analysis (Meays et al., 2004; Scott et al., 2002; Stoeckel and Harwood, 2007). Recently, $(GTG)_5$-PCR has been found to produce more discriminative and complex fingerprint patterns than the others, and can be considered to be a complementary molecular tool for the rapid determination of *E. coli* isolates identity and tracking the non-point sources of fecal pollution (Mohapatra et al., 2007, 2008).

Evaluating studies on livestock management and shellfish growing water quality requires not only an understanding of all the possible sources of fecal pollution in waters, but also the ability to accurately predict the presence of fecal pathogens associated with different host-origins which is central to assessing health risks. However, little is known about annual changes of *E. coli* population concentrations, host sources and correlation with pathogens in the shellfish aquaculture environment of East China Sea.

Therefore, this study was conducted to analyze the temporal genetic variability of fecal *E. coli* by $(GTG)_5$-PCR genomic fingerprinting, and to evaluate the efficacy of this method for differentiating fecal *E. coli* from different domesticated animals farmed around the Xiangshan Bay, a major area for shellfish harvest on the coastline of East China Sea. Moreover, the major zoonotic pathogens correlated with different host-origins were also detected from the contaminated shellfish growing waters.

2. Materials and methods

2.1. Collection of samples

The study site named Xiangshan Bay (Fig. 1) is located at the coastline of East China Sea (121°25′E–120°00′E, 29°05′N–29°46′N) as one of the biggest areas for shellfish harvest in the southeast of China. This bay is a long-narrow, semi-enclosed inlet of the sea with an area of 563.3 km^2, including four major shellfish aquaculture areas cultured clams (site 1), oysters (site 2), mussels (site 3) and scallops (site 4). The low, median and high flow periods are December, March and June, respectively. This region also houses an intensive livestock and poultry breeding industry with a number of farms around the shellfish aquaculture areas.

Table 1 lists the sources of the isolates used in this study, the number of isolates and unique fingerprints obtained from each source from Jan. 2007 to Dec. 2008. Fecal samples of animals were obtained as cloacal swabs from all the domesticated animal farm sites belonged to the area of Xiangshan Bay. Thus, all the types of feces from domesticated animals sampled in this area included swine, chicken, goose, duck, goat, rabbit, cow, sheep and ostrich. The swabs were stored in sterile test tubes and kept at 4 °C until processed, usually within 6 h. On each shellfish aquaculture site, 100 water samples were collected in each of the four periods (Sep. 2009, Dec. 2009, Mar. 2010 and Jun. 2010). Totally, 1600 water samples were collected on 4 sites during different periods using sterilized 500 ml plastic bottles. At each sampling time, 10 samples were collected 2 min apart to obtain a more representative sample and reduce the chances of sampling a fecal plume. Samples were immediately placed in a cooler with ice after sampling and transported to the laboratory for *E. coli* analysis within 24 h of sampling.

Table 1
Animal source groups representing domesticated animals throughout Xiangshan Bay and $(GTG)_5$-PCR DNA fingerprints generated from *E. coli* isolates in the years 2007 and 2008.

Source	No. of samples	No. of isolates	No. of unique fingerprints[a]
Swine	137	212	165
Chickens	178	336	258
Ducks	211	309	231
Geese	147	273	172
Goats	92	171	103
Rabbits	73	142	101
Sheep	83	167	96
Cows	62	108	81
Ostriches	121	204	156
Total	1104	1922	1363

[a] Identical *E. coli* genotypes from each individual animal were removed.