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Detection of *Aeromonas caviae* in the common housefly *Musca domestica* by culture and polymerase chain reaction

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SUMMARY

Aeromonas caviae has been implicated in diarrhoeal disease of livestock and humans. The potential role of houseflies in the epidemiology of this pathogen was investigated by examining the prevalence of *A. caviae* in houseflies collected from two South Carolina farms and one restaurant. Isolation was accomplished by culture of flies in alkaline peptone water followed by identification with *Aeromonas*-specific PCR using novel primers (APW–PCR). All isolates cultured from houseflies were identified as *A. caviae* by biochemical characteristics and direct sequencing ~ 800 bp of the 16S rRNA gene. *Aeromonas caviae* was detected in 78% (272/349) dairy farm flies, 55% (54/99) pig farm flies and 39% (77/200) restaurant flies. Faeces from cows and pigs at the farms also were positive for *A. caviae* (58% and 100%, respectively). The APW–PCR method provided a rapid, convenient way to identify *A. caviae* from faeces and houseflies that contained hundreds of bacterial species.

INTRODUCTION

Mesophilic *Aeromonas* spp. (*A. caviae*, *A. hydrophila*, *A. sobria* – 'aeromonads') have been associated with diarrhoeal disease in both humans and livestock [1, 2]. In some cases, *A. caviae* has been implicated in human enteritis [3] that persisted as chronic diarrhoea for years despite antibiotic treatment [2]. As the most prevalent aeromonad isolated from both normal and diarrhoeic stools (including paediatric specimens) [4–6], *A. caviae* also was the sole enteric pathogen isolated from 14 of 17 paediatric cholera-like diarrhoeal stools [7]. Even so, the actual incidence of this enteric pathogen is probably widely under-detected, since most clinical laboratories cannot accurately identify *Aeromonas* isolates at the species level by use of commercial identification kits and routine methods are not sensitive to detect *Aeromonas* spp. [8].

Aeromonads, many of which produce putative virulence factors [9], are routinely found in water and sewage and survive for months in soil [10]. These bacteria also have been isolated from faeces, bedding and drinking water of healthy cows and pigs [11]. Since both diarrhoeic and healthy horses, chickens, goats, rabbits, pigs and sheep shed aeromonads in their faeces [1, 12], the major mode of aeromonad transmission to humans has been assumed to be faecal–oral, possibly via ingestion of contaminated water. However, their widespread distribution in nature and in agricultural environments suggests the potential for other means of transmission.

The common housefly, *Musca domestica*, encounters faecal flora while feeding and ovipositing on excrement. Accordingly, houseflies are commonly implicated as agents in the spread of gastrointestinal pathogens including *Klebsiella* sp., *Salmonella* sp., *Escherichia coli*, *Proteus* sp., *Shigella* sp., and *Campylobacter* sp. [14–20]. Further, houseflies are capable of harbouring *A. hydrophila* [10, 16, 21], although their epidemiological role in transmission of this aeromonad or related species has not been investigated. Here we report the prevalence of *A.*

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caviae in houseflies as determined by both culture and PCR techniques.

MATERIALS AND METHODS

Sample collection

Houseflies (*Musca domestica*) were captured via vacuum entrapment from Clemson University dairy and pig farms and an upstate South Carolina restaurant (~ 6·3 km from the dairy farm). At the farms, flies were trapped on or around animals, their feed and their faeces. At the restaurant, flies were captured from the kitchen, the public dining room, and near a dumpster in the parking lot. Flies were anaesthetized on-site with ethyl acetate and placed in sterile plastic tubes for transport. For immediate culturing of bacteria, flies were processed within 1 h; remaining flies were frozen at −80 °C for later homogenization. Approximately 5 g of cow or pig faeces was collected from fresh droppings or from faecal grab samples directly from the rectum. Faecal samples were transferred to sterile plastic tubes until cultured. Samples of the animals' feed and bedding also were collected. Food collected from the restaurant's kitchen during normal operation included mayonnaise, salad mix, calabash shrimp, flounder, chicken, shrimp salad, deli meat, hamburger, buttermilk and coleslaw. All foods were in bins or buckets at room temperature (~ 23 °C) and were uncooked. Five spatially-distinct samples from each food bin were pooled for analysis.

Culturing and identification of *Aeromonas* sp. from samples

A single fly, 1 g faeces, or an environmental or food sample, was homogenized individually in sterile water. Five ml alkaline peptone water (APW, pH 8·6, with 30 mg/l ampicillin and 3 mg/l vancomycin) was inoculated with 0·5 ml fly or faeces homogenate and incubated overnight at 37 °C. One loopful of growth was streaked on Ryan's *Aeromonas* agar (AA, Oxoid, Ltd, Basingstoke, Hampshire, England) with 20 mg/l ampicillin and 5 mg/l irgasan. Green colonies with dark green centres were selected and transferred to ampicillin (30 mg/l) blood agar containing 5% sheep or rabbit blood. Motile, oxidase positive colonies were selected and transferred to API20E strips (BioMérieux, Inc., Hazelwood, MO) for species identification. Isolates also were subcultured on MacConkey agar to test for lactose fermentation. Two ml of the remaining homogenate was centrifuged at 14000 ***g*** for 5 min, and DNA was extracted from the bacterial pellet for PCR. Standard membrane filtration was used for enumeration of bacteria in water samples [22]. Feed, bedding and soil samples were vortexed in 50 ml sterile saline for 10 min. Particulates were trapped on a 0·45 μm filter (Millipore, Bedford, MA). The filter was placed on AA and incubated overnight at 37 °C. Colonies were selected and identified using the same methods as for fly and faeces cultures.

Dissection of houseflies

To determine the location of *A. caviae* in or on houseflies, external surfaces and internal organs were cultured separately. Flies were placed in 5 ml sterile APW and vortexed for 2 min. After removal of the fly, APW was incubated overnight at 37 °C. The fly was then immersed in 70% ethanol for 5 min. Surface-decontaminated flies were dissected longitudinally through the thorax and abdomen. The digestive system was removed with a sterile, curved dental pick and placed in APW. After 24 h incubation, a loop of growth was transferred to AA for species identification; the remaining liquid culture was retained for molecular analysis.

Molecular analysis of *A. caviae* from samples

DNA was extracted from cultured isolates, APW growth, and whole-fly homogenate (uncultured, frozen flies) using the CTAB extraction method [23]. DNA was amplified by PCR using one of two primer sets (Table 1). Genus-specific primer set 1 was initially used to screen flies for *Aeromonas* spp., while primer set 2 was designed to be specific for *A. caviae* and *A. jandae* only.

Primers were sufficiently sensitive and specific to amplify bacterial DNA from samples of mixed origin (e.g. samples containing *Aeromonas* DNA as well as fly DNA and other bacteria such as *Pseudomonas* spp., *Proteus* spp. and *Enterobacter* spp.). Primers were designed by aligning *Aeromonas* spp. 16S rRNA sequences (acquired from GenBank®) with close relatives of the gamma subdivision of the proteobacteria (e.g. *Pseudomonas* spp. and the Enterobacteriacae) using Megalign® software (DNASTAR, Inc., Madison, WI). Candidate primers were tested in PCR simulation using Amplify 1.2® (W. Engels, Madison, WI).

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Table 1. Aeromonas-*specific primers used for PCR*

Primer set	Sequences	Amplicon size
1 (Fd/Rev)	Fd: 5′-TAGCTTGCTACTTTTGCCGG-3′ Rev: 5′-GACACAGGAACTCTGCACCG-3′	~ 800 bp
2 (Fd/ACJ)	Fd: 5′-TAGCTTGCTACTTTTGCCGG-3′ ACJ: 5′-CACAGCCAGCAGRTATTAGCYACT-3′	~ 450 bp

Master*Taq*® DNA polymerase (Eppendorf Scientific, Westbury, NY) was used for all PCRs according to manufacturer's directions and included '*Taq*Master® PCR Enhancer' to improve yield and specificity of target sequences. Samples were incubated in a Techne Genius® DNA thermal cycler for 2 min at 94 °C, then cycled 35 times at 94 °C for 15 s, 56 °C for 15 s and 72 °C for 20 s, with a final extension at 72 °C for 2 min. Eight μl of product was added to 1 μl ethidium bromide (5 ng/μl) and 1 μl bromophenol blue and run on a 1·5% agarose gel (SeaKem LE, FMC Bioproducts) for 45 min to 1 h at 70V. PCR products were visualized with ultraviolet light. Images of gels were taken with a Kodak DC 120 digital camera. Ethanol-precipitated PCR products were sequenced using ABI PRISM™ dye terminator cycle sequencing (PE Biosystems) following manufacturer's instructions for PCR. Sequences from PCR amplicons of farm isolates were aligned with each other and *Aeromonas* GenBank® sequences using BLASTn and MegAlign® (DNASTAR, Inc., Madison, WI).

RESULTS

Aeromonas caviae was identified using API 20E strips as this species differs from other aeromonads by being Voges-Praskauer and lysine decarboxylase negative. Further, although this species is typically lactose-positive on MacConkey agar, most isolates were lactose-negative. All species isolated from the flies were non-haemolytic on both sheep- and rabbit-blood agar.

Aeromonas caviae was detected by PCR of DNA extracted from whole, frozen flies and DNA from bacteria cultured from homogenized flies. Early in the study, flies were either cultured in APW and plated on AA to detect viable bacteria, or flies were frozen for total DNA extraction and subsequent *Aeromonas*-specific PCR. PCR was more sensitive in detecting *A. caviae* associated with houseflies at both farms; 66% and 57% of flies were PCR-positive while 30% and 5% were culture-positive, at the dairy and swine farms respectively.

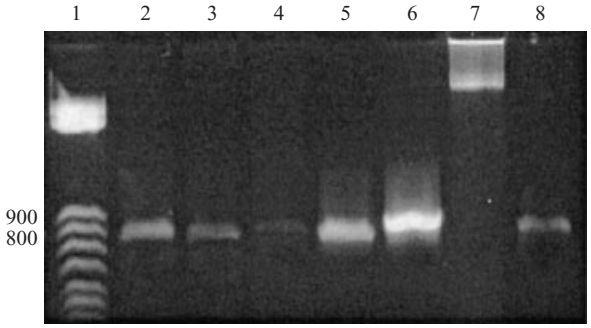

Fig. 1. PCR using *Aeromonas* genus-specific primer set 1. Lane 1, 50 bp ladder (Sigma); lanes 2–4, *Musca domestica* whole fly extract from dairy farm; lane 5, *M. domestica* from pig farm; lane 6, *A. caviae* isolate 'C' from housefly; lane 7, *P. aeruginosa* (negative control); lane 8, *A. caviae* isolate 'A' from housefly. Products were ~ 800 bp.

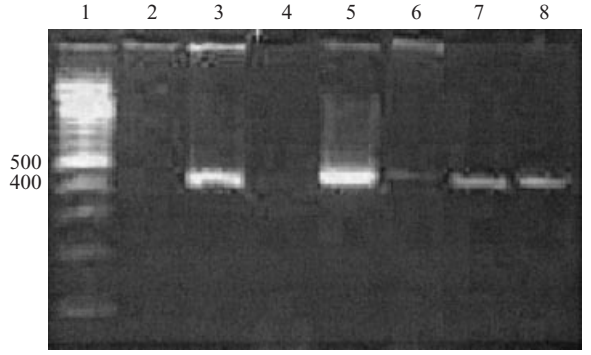

Fig. 2. PCR using *Aeromonas* primer set 2 on cultured viscera and external washings from houseflies collected at dairy farm, 5/17/00. Lane 1, 100 bp ladder (New England BioLabs); lanes 2, 4 and 6, external surface washings; lanes 3, 5 and 7, viscera respective to external washings; lane 8, positive control. Amplicons were the expected size of ~ 450 bp.

In subsequent collections, molecular and culturing techniques were combined (APW–PCR), and flies were considered *A. caviae*-positive if product was amplified from DNA isolated from APW bacterial growth. Primer set 1 amplified a product ~ 800 bp in length (Fig. 1) while primer set 2 amplified ~ 450 bp (Fig. 2) from DNA extracted from whole flies and cultured flies and faeces. Though primer set 1 was genus-specific and was shown by PCR simulation

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Table 2. *GenBank® accession numbers for partial sequences of* 16*S rRNA gene of* A. caviae *isolated from houseflies*

Isolate	GenBank® accession number
FlyA (whole fly culture*)	AF170282
FlyC (whole fly culture)	AF170283
Fly2 (whole fly culture)	AF210253
Fly4 (whole fly culture)	AF210254
Fly5 (whole fly culture)	AF210255
Fly1 (whole fly extraction†)	AF210251
Fly12 (whole fly extraction)	AF210252
CG3 (fly gut culture)	AF281628
CO5 (fly surface culture)	AF281629
P5 (whole fly culture)	AF281630
PG3 (fly gut culture)	AF281631

* Whole flies, surfaces or viscera were cultured first and DNA was extracted from *A. caviae* isolates.
† DNA was extracted from whole-fly homogenate without prior culture.

using Amplify 1.2® (W. Engels, Madison, WI) to have the capability of amplifying other aeromonads, sequence analysis revealed that only *A. caviae* DNA was amplified using either primer set during this study. Partial sequences of 16S rDNA from some isolates are available in GenBank under accession numbers listed in Table 2. Sequences aligned greater than 98% with other *A. caviae* 16S rDNA sequences, which further confirmed the identity of the fly isolates. Overall, *A. caviae* was detected in 78% (272/349) dairy farm flies, 55% (54/99) swine farm flies and 39% (77/200) restaurant-associated flies. Notably, all restaurant flies that were positive for *A. caviae* were captured in the kitchen (77/150); none from the dining room or dumpster was positive (0/50). All food samples were positive for *A. caviae* by APW–PCR. Pooled samples of the municipal water supply were PCR-negative. *Aeromonas caviae* also was detected in 58% (17/29) of cow grab samples, 100% (3/3) of fresh pig faecal samples and consistently from pooled samples (five randomly chosen samples per pool) of the animals' drinking water, bedding, feed and soil.

Aeromonas caviae was cultured both from viscera and from external surface washings of dissected flies. APW–PCR using primer set 2 amplified *A. caviae* 16S rDNA (Fig. 2) from 47% (14/30) cultured gut and 57% (17/30) cultured external surfaces. Primer set 1 also amplified product from the same positive samples that were used for subsequent sequence analysis (data not shown). No significant difference was observed between the presence of bacteria on the surface or within the flies ($P < 0{\cdot}05$; Fischer's exact test).

DISCUSSION

Although *A. hydrophila* and other aeromonads (unidentified species) have been isolated from houseflies [10, 16, 21], to our knowledge, this is the first report of *A. caviae* in houseflies. Recently, Sulaiman et al. [24] isolated *A. hydrophila* from blowflies (*Chrysomya megacephala*), houseflies and face flies (*Musca sorbens*) collected from market areas and dumps in Malaysia, but these investigators were unable to isolate *A. caviae* from houseflies or face flies, with isolation from just two blowflies. However, all of these studies used only standard culturing methods. In the current study, routine attempts to culture bacteria from mixed sources resulted in potential underdetection due to overgrowth of competing bacteria on agar plates or an inability to identify bacteria to species since only biochemical characters are available for evaluation. Therefore, the methodology used in this study combined culturing and molecular techniques, which significantly increased the sensitivity and specificity of detection of *A. caviae* among the numerous species of bacteria that exist in and on houseflies. This technique (APW–PCR) had two advantages over culturing or PCR alone. First, because bacteria multiplied in APW, amplification of total bacterial DNA increased sensitivity of detection. Also, selection for motile, viable bacteria (aeromonads and others) was accomplished by removing the top 1·5–2 ml of APW for DNA extraction without agitating bacteria settling in the bottom of the test tube.

Houseflies have long been implicated as vectors and reservoirs for enteric pathogens that they ingest or encounter during coprophagia. Although not the primary mode of transmission, *Musca domestica* transmits *Campylobacter* sp. to chickens [17] and *Corynebacterium* sp. in dairy cattle herds [25]. Flies can transmit bacteria either by contamination when they alight on the animals' food or water or by being eaten when the animal feeds. In this study, flies were observed moving freely from manure to the animals' food bins. Also, animals were observed eating both live and dead flies. Transmission of bacteria by ingestion of flies or their excreta may be possible even after the flies are dead and dried out. Rosef and Kapperud [26] found *Campylobacter* sp. remained

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viable in the excreta and on the surface of flies that had been desiccated for several days. The existence of various routes for flies to transmit bacteria suggests houseflies served as a source of *A. caviae* for the animals at the farms.

Faeces were one probable source of the bacterium for houseflies at the farms since *A. caviae* was frequently isolated from animal manure and has been cultured from livestock faeces in other studies [10, 11]. The presence of *A. caviae* in flies at the restaurant was significant because of potential contamination of food. Although all food items sampled from the restaurant kitchen were positive for *A. caviae*, whether the food served as a source of the bacterium for the flies or the food was contaminated by flies could not be determined. However, it is probable that the food served as a source of contamination for the flies, since only the kitchen flies (and not the dumpster or dining room flies) were positive. Further, one or a few of the foods may have served as a common source of contamination from which the flies disseminated bacteria. Aeromonads have been reported from food items in other studies [27, 28]. Even though some foods in this study arrived fresh and others were frozen or refrigerated, a number of strains of aeromonads are psychrotrophic, not only surviving but also growing in foods at low temperatures [29–30]. Regardless of the source, contamination of flies and food by *A. caviae* has significant epidemiological implications.

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