Helminth–bacteria interaction in the gut of domestic pigeon *Columba livia domestica*

Debraj Biswal · Anadi Prasad Nandi ·
Soumendranath Chatterjee

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Abstract The present paper is an attempt to study the interaction between the helminth parasite and bacteria residing in the gut of domestic pigeon, *Columba livia domestica*. Biochemical and molecular characterization of the gut bacterial isolate were done and the isolate was identified as *Staphylococcus* sp. DB1 (JX442510). The interaction of *Staphylococcus* sp. with *Cotugnia cuneata*, an intestinal helminth parasite of domestic pigeon was studied on the basis of the difference between ‘mean worm burden’ of antibiotic treated infected pigeons and infected pigeons without any antibiotic treatment. The ANOVA and Tukey tests of the data obtained showed that antibiotic treatment reduced the mean worm burden significantly. The biochemical properties of *Staphylococcus* sp. DB1 (JX442510) also showed a mutualistic relationship with the physiology of *C. cuneata*.

Keywords *Staphylococcus* sp. · *Cotugnia cuneata* · Antibiotic treatment · Mean worm burden · Mutualistic relationship

Introduction

The animal gut exhibits a complex ecosystem consisting mainly of bacteria, protozoa, fungi or the microbes (or microfauna) and the endoparasites mainly the helminths that make up the macrofauna. The species composition of the gut microbiota varies greatly between individuals and

each individual harbors a unique collection of bacterial species, which may however change with time (Bäckhed et al. 2005; Qin et al. 2010). The composition of the microbial flora of the host gut is also dependent partly on the environmental conditions of the host, its feeding and foraging habits, the nutritional status, age-sex factor and the immunological status of the host (Mazmanian et al. 2005; Zoetendal et al. 2001; Petkevicius et al. 2007).

Animals are always in an interactive relation with the environmental microbiota (Sekirov et al. 2010) which has a tendency of getting colonized within the GI tract (Ley et al. 2008). The intestinal microbiota surprisingly maintains a remarkably stable composition (Kunz et al. 2009). However, the mechanism by which the equilibrium of these microbes is maintained at different levels of the GI tract is yet to be deciphered (Ley et al. 2008; Kunz et al. 2009). The GI tract of the animals after their birth are subjected to colonization by bacteria (Morelli 2008) which play many beneficial roles, the most important one being the resistance against invasion by pathogenic bacteria (Bancroft et al. 2012; Kamada et al. 2012). Colonization of commensal bacterial flora has important effects on the nutritional as well as the defensive functions of the gut by modulation of gene expression (Hooper and Gordon 2001). Considering all these complexities of the gastrointestinal tract, it seems that the maintenance of homeostasis in the gut environment is crucial to the maintenance of good health of the host (Sekirov et al. 2010; Clemente et al. 2012) while sudden changes in the gut microbiota can lead to dire consequences (Palming et al. 2006). The aberrant physiological changes and poor host defense occurring in the absence of microbiota can be reversed by reintroduction of bacteria (Hooper et al. 2003). The concept “hygiene hypothesis” (Wold et al. 1998) was broadened a bit further by Yazdanbakhsh and Matricardi (2004) by including the

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D. Biswal · A. P. Nandi · S. Chatterjee (✉)
Parasitology and Microbiology Research Laboratory,
Department of Zoology, The University of Burdwan,
Burdwan 713104, West Bengal, India
e-mail: soumen.microbiology@gmail.com

helminth parasites or the “macrofauna” of the gut. Helminths play a key role in the evolution and selection of interleukin genes (Bäckhed et al. 2005). The co-existence of the commensal gut-bacteria and the intestinal helminths over a long period established an association between them through co-evolution (Hayes et al. 2010). Studies so far have mostly been concentrated on the symbiotic relationships between the intestinal microbial populations and the host while the interactions between the microfauna and the macrofauna have long been neglected (Hayes et al. 2010). Intimate relationships between the metazoa and the bacteria have been well documented in filarial worms and the endosymbiont *Wolbachia* (Taylor et al. 2005). Gut-microbiota of Bumble Bee has also been found to confer resistance to the major parasite, *Crithidia bombi* (Koch and Schmid-Hempel 2011). Many other recent studies have pointed out the fact that microfauna, macrofauna and host immune system work in a well integrated and balanced system that has been developed by co-evolution over a long period of time (Walk et al. 2010; Berrilli et al. 2012).

Recent studies in this field (on vertebrates) have been limited only to mammals using mice and pigs as model organisms since the physiology of humans are nearly similar in these animals (Dawson 2011). Other animals, specially the birds have long been neglected. The present study is an attempt to unravel helminth-bacteria interaction in domestic pigeon, *Columba livia domestica*.

Materials and methods

Isolation of resident bacterial flora

The entire digestive tract of the pigeon, selected randomly from different lofts, was dissected out and its surface was sterilized by rubbing 70 % alcohol. Under sterile conditions, the intestine was cut open and the gut contents, specially the inner surface of the intestine was scrapped off and diluted in sterile distilled water. Pour plates and spread plates were made with nutrient agar (peptone–beef extract–NaCl–agar at 5:3:3:18 g/l, pH 7.4) and incubated in a B.O.D incubator at 37 ± 0.1 °C for 24 h. Pure culture was maintained for only the resident bacterial isolate found all through the year.

Morphological and biochemical characterization

The morphological characteristics of the isolate were studied by Gram-staining methods followed by staining with malachite green for detecting the presence of endospores. SEM photographs were also taken at different magnifications. The size of the bacterial cells was determined by micrometry. The isolate was subjected to a

number of biochemical tests like indole, methyl red, Vogues-Proskauer, citrate utilization, catalase, nitrate reduction, oxidase, urease, coagulase and blood haemolysis tests. Fermentation tests were carried out taking Lactose, Glucose, Sucrose, Trehalose, D-arabinose, Glycogen, Raffinose and Dextrose as carbon source. The isolate was also grown on TSI agar (triple sugar iron agar) and the change in colour was noted for. Starch, gelatin and lipid hydrolysis tests were done to detect the enzymatic activity. NaCl tolerance tests were also performed by growing the bacteria in 2, 4, 6, 8 and 10 % NaCl dissolved in nutrient broth. pH tolerance tests were done by growing the bacteria in nutrient broth with pH adjusted at 2, 4, 6, 8 and 10. All the tests were done following Pelczar et al. (1957); Sneath (1986); Collee and Miles (1989) and Lacey (1997). Antibiotic sensitivity tests were performed using antibiotic discs (Himedia, Germany) and the results were recorded after measuring the zone of inhibition obtained and comparing with standard charts (Brown 2007). The tests were carried out for rifampicin (5 µg/disc), chloramphenicol (30 µg/disc), ciprofloxacin (5 µg/disc), tetracycline (30 µg/disc), vancomycin (30 µg/disc), doxycycline hydrochloride (30 µg/disc), gatifloxacin (5 µg/disc), ampicillin (10 µg/disc), gentamicin (10 µg/disc), kanamycin (30 µg/disc) and levofloxacin (5 µg/disc).

Molecular characterization

Studies on the phylogenetic affiliations of the bacteria were carried out by 16S rRNA gene sequence analysis. Nucleotide BLAST (BLASTN) was carried out to investigate the most similar sequences from NCBI database. The sequence data was finally aligned using the ‘ClustalW Submission Form’ and analyzed by ClustalW (Thompson et al. 1994). Evolutionary distances were calculated using the methods of Jukes and Cantor (1969) and the topology was inferred using the ‘neighbour-joining’ method (Saitou and Nei 1987). The phylogenetic tree was prepared following Tamura et al. (2007) in order to assign the taxonomic affiliations of the bacteria under investigation.

Helminth–bacteria interaction

The helminth considered in this experiment was a cestode, *Cotugnia cuneata*, an intestinal endoparasite of domestic pigeons (Biswal et al. 2012, 2013).

A total number of 80 infected pigeons (confirmed by stool-test) were selected randomly from different lofts and divided into eight equal groups with 10 pigeons in each group. Out of these, four groups were fed food-grains treated with chloramphenicol (to which the isolate was found to be most sensitive). The remaining four groups

were kept as control and were fed with normal diet, without any antibiotic treatment. The mean worm burden (with respect to the number of *Cotugnia cuneata*) of both treated and control birds was checked after 10, 20, 30 and 40 days by sacrificing each group on the 10th, 20th, 30th and 40th day of the experiment. The whole experiment was replicated five times and the results were noted down.

The mean worm burden was calculated by the following formula:

Mean Worm Burden = Total no. of worms obtained from the group of infected birds/Total no. of infected birds present in the group (=10, in this study).

The data obtained were subjected to statistical analyses following Zar (1999). Analysis of variance (ANOVA) was employed to determine:

- a. The effect of the antibiotic-treatment on the mean worm burden of the treated bird groups by comparing them with the control bird groups, and
- b. The effect of the duration of the antibiotic-treatment on the mean worm burden of the treated bird groups.

Tukey test was used for multiple comparisons of means as and when needed.

Results

The bacterial isolate was Gram-positive and round shaped (Table 1; Fig. 1). No endospore was found. It was identified as *Staphylococcus* sp. DB1 (JX442510) by biochemical tests (Table 1) and gene sequencing (Fig. 2). The isolate was positive for catalase, nitrate reduction, urease, methyl red and starch hydrolysis tests and showed negative reaction indole, Vogues-Proskauer, oxidase, citrate utilization, fat hydrolysis and gelatin hydrolysis tests. The isolate was negative for coagulase and blood haemolysis tests which are usually found positive in pathogenic strains of *Staphylococcus* sp. The isolate was grown in Triple Sugar Iron agar (TSI agar) resulting in a yellow slant and yellow butt showing that it was a lactose fermenter. The isolate could produce acid from carbon sources like trehalose, glycogen, raffinose, D-arabinose, sucrose, lactose, glucose and dextrose but could produce gas from none. The isolate showed optimum growth at pH 7 and 8. NaCl tolerance tests revealed that it could grow in NaCl concentrations of 2 and 4 % while the growth declined from 6 to 10 % with least growth at 10 % NaCl solution in Nutrient Broth. Antibiotic sensitivity tests showed that the isolate was sensitive to recommended doses of chloramphenicol (30 µg/disc), ciprofloxacin (5 µg/disc), doxycycline hydrochloride (30 µg/disc), gentamicin (10 µg/disc), levofloxacin (5 µg/disc), tetracycline (30 µg/disc) and gatifloxacin (5 µg/disc) while it was resistant to ampicillin

(10 µg/disc), rifampicin (5 µg/disc), vancomycin (30 µg/disc) and kanamycin (30 µg/disc).

The results of detailed pH-tolerance, NaCl-tolerance, biochemical, fermentation, hydrolysis and antibiotic sensitivity tests have been summarized in Table 1. The phylogenetic tree and nucleotide composition of 16S rRNA gene sequence of *Staphylococcus* sp. DB1 (JX442510) has been shown in Figs. 2, 3 respectively.

Table 2 clearly shows that the antibiotic treatment caused significant changes in the mean worm burden of infected pigeons after 20, 30 and 40 days of treatment in comparison to control groups. After 10 days of treatment however, no significant changes were recorded in respect to the mean worm burden (Table 2). Comparisons between the treated and untreated group shows that the mean worm burden after 30 and 40 days of treatment was not significant (Table 2). The reduction of mean worm burden in the treated group showed a sharp decline between the 10 and 20 days treatment-duration (Fig. 4) while the 30–40 days treatment-duration showed only an insignificant drop (Fig. 4).

Discussion

The alimentary canal of pigeon is based on the same general plan of that of other vertebrates (Vispo and Karasov 1997). It consists of the oesophagus, crop, proventriculus, gizzard, small intestine, caeca, large intestine and the cloaca. Earlier reports show that microbes are found throughout the alimentary canal of birds but are usually higher in regions which are relatively stationary and the food materials are retained for a longer period thereby providing ample conditions for microbial growth (Vispo and Karasov 1997). According to them, the small intestine of birds is not a suitable site for colonization by microbes since the food is not retained for long in this part of the GI tract (Vispo and Karasov 1997). However, Savage (1970) reported the presence of some filamentous bacteria in the small intestine of domestic chickens. Herd and Dawson (1984) and Buchsbaum et al. (1986) conducted separate studies on Emu and Geese, respectively, and concluded that microbial fermentation in the small intestine plays a significant role in these birds. There is however, a dearth of information on the microbial flora from the gut of pigeon. Some recent studies have reported the presence of *Lactobacillus agilis* as the main component of the pigeon crop flora (Baele et al. 2001). *Enterococcus columbae*, *E. cecorum*, *E. faecalis*, *E. faecium*, *E. gallinarum*, *E. casseliflavus*, *Streptococcus alactolyticus* and *S. gallolyticus* have been reported from the intestine of pigeons (Devriese et al. 1990, 1993; Baele et al. 2002). Examination of the gut of infected pigeons showed that the worms are found mainly

Table 1 Morphological, physiological and biochemical characteristics of *Staphylococcus* sp. DB1 (JX442510)

Characteristic/test	Observation	Characteristic/test	Observation	
Morphology	Round shaped	Carbon-source	Acid	Gas
		Trehalose	+	–
		Glycogen	+	–
		Raffinose	+	–
		D-arabinose	+	–
		Sucrose	+	–
		Lactose	+	–
		Glucose	+	–
		Dextrose	+	–
Diameter (mean $\pm$ SD)	0.78 $\pm$ 0.11 μ m			
Gram-staining	+ve			
Endospore staining with malachite green	–ve			
pH tolerance test	Optimum growth at pH 7 and 8. Growth declines on either side of this			
NaCl tolerance test	Grown in 2, 4, 6, 8 and 10 % NaCl solution. Growth declines from 6 to 10 % with least growth at 10 %	Biochemical tests	+ve	–ve
		Indole		–
		Methyl red	+	
		Voges-Proskauer		–
		Citrate		–
		Catalase	+	
		Nitrate	+	
		Oxidase		–
Grown in TSI (triple sugar iron) agar	Growth came with yellow slant-yellow butt	Urease	+	
		Coagulase		–
		Blood Haemolysis		–
Hydrolysis test	+ve			
Starch	+			
Fat				–
Gelatin				–
Antibiotic discs (μ g/disc)	Sensitive (S)	Resistant (R)	Antibiotic discs (μ g/disc)	Sensitive (S) Resistant (R)
Chloramphenicol (30)	S	–	Ampicillin (10)	– R
			Rifampicin (5)	– R
			Vancomycin (30)	– R
			Kanamycin (30)	– R
Ciprofloxacin (5)	S	–		
Doxycycline				
Hydrochloride (30)	S	–		
Gentamicin (10)	S	–		
Levofloxacin (5)	S	–		
Tetracycline (30)	S	–		
Gatifloxacin (5)	S	–		

in the small intestine (Biswal et al. 2012, 2013). Therefore the microbial population of this region was given priority and taken into account.

In the current study, *Staphylococcus* sp. DB1 (JX442510) was found to be the most prevalent flora. The strain of *Staphylococcus* sp. obtained was non-pathogenic and was lactose fermenter (as evident from biochemical tests). The phylogenetic tree showed that *Staphylococcus* sp. DB1 (JX442510) branched with *Staphylococcus*

piscifermentans (AF041359) with 72 % bootstrap support (Fig. 2). Earlier report on non-pathogenic strains of *Staphylococcus* sp. show that they have mainly been isolated from fermented fish (Tanasupawat et al. 1992) or from some food products (Hajar and Hamid 2013). The lactic acid strains of *Staphylococcus* sp. have also been reported from healthy dog faeces, which, Štetina et al. (2005) concluded to have arrived from fermented food residue in dog feed. Whatever may be, there seems a dearth

Fig. 1 Scanning electron micrograph of *Staphylococcus* sp. DB1 (JX442510). **a** 8000 $\times$, **b** 20,000 $\times$

Fig. 2 Neighbour-joining tree constructed on the basis of 16S rRNA gene sequences of *Staphylococcus* sp. DB1 (JX442510) along with 16S rRNA genes of other bacteria retrieved from NCBI database

in the reporting of this particular species in food and food-products (Probst et al. 1998). Considering earlier reports, it may be hypothesized that this particular non-pathogenic and lactic acid strain of *Staphylococcus* sp. may have gained entry into the pigeon gut via food materials and may have co-existed there for a long period and co-evolved as a prominent gut flora. Vispo and Karasov (1997) in their review had stated that microbial growth occurs preferably

in those regions of the alimentary canal where there is relative stasis, as stated earlier. Considering this, the intestine of pigeon is not a very favourable site for bacterial growth. This was fortified in the present study since the microbial flora obtained from these regions of uninfected pigeons indeed showed a very poor density. However, the bacterial growth was found to be denser in infected pigeons. The reason may be linked to the fact an intestine

Fig. 3 Nucleotide composition of 16S rRNA gene sequence of *Staphylococcus* sp. DB1 (JX442510)

Table 2 Results of ANOVA and Tukey tests of the mean worm burden (Means of five replications $\pm$ SE) of domestic pigeons (control and treated)

Duration of treatment (days)	Mean worm burden	
	Control	Treated
10	11.6 $\pm$ 0.24 ^{ab}	11.38 $\pm$ 0.26 ^{ab}
20	11.36 $\pm$ 0.16 ^a	6.58 $\pm$ 0.34 ^c
30	11.32 $\pm$ 0.17 ^a	4.00 $\pm$ 0.21 ^{df}
40	11.4 $\pm$ 0.16 ^a	3.76 $\pm$ 0.17 ^{ef}

Means followed by different alphabets differ significantly, $P < 0.001$

Fig. 4 Mean worm burden of control and antibiotic treated domestic pigeons

stuffed with helminths provides obstacles to the free flow of food materials (like a sort of traffic-jam) and results in regions of relative stasis. These previously unfavourable sites are thus converted into favourable sites of bacterial growth thereby supporting the view of Vispo and Karasov (1997). However, these seemingly simple events may not be that simple at all. Some recent studies have pointed out the fact that intestinal helminths produce molecules that may cause a shift in the habitats of gut-microbiota (Berrilli et al. 2012). Thus, it may be hypothesized that *C. cuneata*

inhabiting the pigeon-gut may be playing a key role in affecting the bacterial population. The bacteria produce acid by fermentation of food products (Table 1) that may be utilized by the cestode as a source of nutrition and energy (Smyth and McManus 2007; Biswal et al. 2012). The oxygen generated by catalase activity may also be utilized by the cestode as a source of oxygen in the relatively anaerobic conditions of the pigeon gut (Smyth and McManus 2007). The bacteria is also able to hydrolyze starch which forms an important part of pigeons diet. The products of starch hydrolysis may be utilized by *C. cuneata* for energy production, which is quite usual as they absorb simple carbohydrates (Smyth and McManus 2007). The synergistic relationship between the bacteria and the cestode became more clear from the results of the experiment with the antibiotic treatment of infected pigeons. Though the treatment did not cause significant changes in the mean worm burden after 10 days of treatment (Table 1), the changes became prominent after 20 days of treatment. The experiment also showed some surprising results since the mean worm burden of the treated groups after 30 days and treated groups after 40 days of treatment did not differ significantly (Table 1). The means-plot as shown in Fig. 4 makes the results much more prominent as the curve for the treated group drops down while the curve for the control group remains stable.

These results point out some sort of cascade reactions closely co-related with the gut-microbe-helminth metabolic network. This field needs further elaborative work to find out the exact reasons for such variations as the antibiotic treatment in infected pigeons resulted in a significant reduction of the mean worm burden. The result is similar to that of Hayes et al. (2010). By several experiments using mice (infected with *Trichuris muris*) and feeding them with antibiotics (Enrofloxacin) Hayes et al. (2010) had observed that it resulted not only in the depletion of the intestinal microflora but also resulted in a reduced worm burden as compared to that of conventional untreated mice populations. All these observations reveal the fact that the cestode may exploit the gut-microbiota for its peaceful co-existence in the pigeon-gut. This bacteria-cestode interaction indicates a symbiotic relation between the two which is supported by many other similar documentations.

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