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# Antibiotic Resistance and Genetic Diversity of *Escherichia coli* Isolated from Indonesian Monitor Lizards (*Varanus* spp.)

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**It has been long known that bites from monitor lizards (Sauria: Varanidae) may cause severe infections and eventually lead to lethal bacteremia. It is generally assumed that these infections are caused by microbial organisms living in the mouth cavity of monitors. Studying microbial diversity of the mouth cavity from samples taken from Indonesian monitor species, we obtained 12 *Escherichia coli* isolates, which showed resistance to ampicilin. Genetic diversity analysis employing pulsed field gel electrophoresis (PFGE) of 9 isolates revealed 7 different *Spe*I schizotypes. The other three isolates produced smear, degraded DNA banding pattern, possibly caused by endonuclease activity. UPGMA analysis of the *Spe*I-schizotypes obtained from the *E. coli* isolates revealed three clusters. Two of the clusters contained isolates from the species *V. salvator* and *V. yuwonoi*, respectively. The third cluster comprised *V. caerulivirens* and *V. salvator*. Whereas the first two clusters showed a clear reference to species and localities, the third cluster may have been a result of cross-contamination from the cages where the specimens were kept or the food administered. Physiological assays employing Microbact® 24E(12E/12A+12B) and DNA profiling analysis indicated that each of the *E. coli* isolates constitutes a normal element of the microbiota occurring in the mouths of the monitor species studied.**

Key words: ampicilin resistance, *Escherichia coli*, schizotyping, *Varanus* spp.

Microorganisms are possibly the most diverse group of organisms on earth. At the same time not even the order of magnitude of the species numbers is known (Heywood 1995). They occupy a vastly greater range of habitats and microhabitats than macroorganisms and occur wherever life is thermodynamically possible. Microorganisms can live even under extreme environmental conditions such as temperatures over 100°C and below the freezing point, high salinity, high pressure, and high acidity. They may also live in other organisms such as plants and animals, either as pathogenic agents or as normal microbiota (Brock & Madigan 1991). One genus of wall-less bacteria living in insect guts (*Spiroplasma*) may even be the largest genus on earth with over one million species (WCMC 1992). Enormous numbers of these bacteria living as mutualists or commensals in other organisms have never been cultured. Moreover, very little is yet known of the biological functions of bacteria or microorganisms living in environments provided by their host organisms. In addition, few studies have screened the natural occurrence of microorganisms and their diversity in free-living species.

The starting point for our study was the fact that it was repeatedly reported that animals or humans bitten by monitor lizards (genus *Varanus*) showed serious infections, occasionally even causing casualties from the resulting bacteremia. For some time it was assumed that the larger species of *Varanus* posses poisonous bites (Auffenberg 1981). Auffenberg (1981), in his famous study of the Komodo dragon (*Varanus komodoensis*), identified four bacterial species from the buccal bacterial flora sampled from live specimens. These were *Proteus morgani*, *P. mirabilis*, *Providencia* sp., and *Staphylococcus* sp. Auffenberg assumed that infections following bites by the Komodo dragon were caused by species of *Proteus* and *Providencia*, which were discharged into the bloodstream of the victim and finally caused bacteremia. Producing such severe and septic wounds may have an important ecological function as the prey becomes more vulnerable to a second attack or, if a bite is lethal, the specimen is already prey for a scavenger species like the Komodo dragon.

To get a better understanding of the role of the buccal microbial communities found in monitors, we obtained bacterial samples from different species occurring in different regions and in different habitats within Indonesia. Bacteria found were also screened for their resistance to certain antibiotics, since there is a scarcity of data on the occurrence of antibiotic resistance genes in bacteria in their natural environment (Desselberger 1998). On the other hand, many reports describe community-isolated bacteria such as *E. coli*, from hospitals which showed increasing resistance to amoxicillin, products that contain trimethoprim and sulfamethoxazole, ampicillin, and clavulanic acid as well as ampicillin and sulbactam (Neu 1992). Quite recently, bacterial resistance to several other antimicrobial agents has also increased (Desselberger 1998). In view of

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these trends the detection of new resistance genes has become even more important. In this study we analyzed genetic diversity of *E. coli* isolated from buccal microbial communities of monitor lizards (*Varanus* spp.) which may be the "normal" microbiota living in the mouth cavity of monitors.

## MATERIALS AND METHODS

Eleven species of monitors from different regions within Indonesia were sampled (Table 1). Specimens were kept in captivity by a reptile trader in Jakarta. For sampling buccal bacteria, sterilized cotton swabs were used to wipe the teeth and crevices, mouth cavity, and to collect saliva (Figure 1). Each sample was put into 2 ml sterile 0.85% physiological NaCl solution.

For the isolation of the bacteria and for later tests of resistance to antibiotics, approximately 20 µl of each sample was spread onto eosin methylene blue agar (EMB; Difco Laboratories, Michigan) and incubated overnight at 37°C. Quadrant streak methods were used to purify and isolate colonies. The single colonies were further inoculated on EMB agar supplemented with ampicillin (100 µg $ml^{-1}$) to select for ampicillin-resistant colonies. *E. coli* was identified by colony "morphology" such as periphery, surface, shape, mucosity, color, and greenish metallic sheen as well as physiological tests using Microbact® 24 E (12E/12A+12B; Medvet Science Pty. Ltd., Australia).

Intact genomic DNA and restriction digestion was performed as follows. The gel inserts (20 x 10 x 1 mm) were prepared as described previously (Suwanto & Kaplan 1989). Restriction endonuclease digestion was performed in 150 µl of restriction buffer in a 1.5 ml microcentrifuge tube. For each piece of gel insert 10 U of enzyme were used. We used 1 x NEB2 buffer for *Spe*I (New England BioLabs, Beverly, MA). The gel inserts were equilibrated in 150 µl of 1xNEB2 buffer for 15 minutes at 4°C, the buffer was aspirated, the tube was filled with fresh buffer, and the appropriate amount of enzyme was added. Digestion was performed overnight, at 37°C. After digestion, the buffer was aspirated, the gel insert was dialyzed for at least 15 minutes by adding 1.5 ml of 1 x TE buffer (10 mM Tris-Cl pH 8.0; 1 mM EDTA pH 8.0) before placing the gel insert into the running gel. *Rhodobacter sphaeroides* 2.4.1 DNA, digested with *Ase*I (Suwanto & Kaplan 1989) was routinely used as molecular size marker.

We used schizotyping or macrorestriction fragment length polymorphisms (MFLP) employing pulsed field gel electrophoresis (PFGE) to analyze the genetic diversity of the *E. coli* isolates. Schizotyping is a DNA profile analysis employing rare-cutting restriction endonucleases and separation of the resulting DNA fragments employing PFGE (Suwanto & Kaplan 1992). Recently, PFGE has been used to analyze genetic diversity in *Brucella* strains (Allardet-Servent *et al.* 1988), *E. coli* (Bergthorsson & Ochman 1995), *Listeria* (Howard *et al.* 1992), *Streptomyces ambofaciens* (Leblond *et al.* 1990),

Table 1. Monitor species sampled, their geographic origin, and diet in captivity. *Varanus caerulivirens* from the Moluccas (Halmahera) has only recently been described (Ziegler *et al.* 1999).

| Species | Origin | Feed |
|---|---|---|
| *Varanus prasinus* | Sorong, Papua | Mice |
| *V. prasinus* | Biak Island | Mice |
| *V. prasinus* | Sorong, Papua | Mice |
| *V. salvator* | Manado, Sulawesi | Chicken |
| *V. salvator* | Manado, Sulawesi | Chicken |
| *V. salvator* | Java | Chicken, mice |
| *V. salvator* | Java | Chicken, mice |
| *V. salvator* | Java | Chicken, mice |
| *V. similis* | Merauke, Papua | Chicken, mice |
| *V. similis* | Merauke, Papua | Chicken, mice |
| *V. similis* | Merauke, Papua | Chicken, mice |
| *V. similis* | Merauke, Papua | Chicken, mice |
| *V. similis* | Merauke, Papua | Chicken, mice |
| *V. timorensis* | Roti Island | Chicken, mice |
| *V. indicus* | Sorong, Papua | Chicken |
| *V. indicus* | Sorong, Papua | Crickets, fish, mice |
| *V. indicus* | Sorong, Papua | Chicken, crickets, mice |
| *V. indicus* | Halmahera | Chicken, crickets, mice |
| *V. indicus* | Halmahera | Mice |
| *V. caerulivirens* | Halmahera | Mice |
| *V. caerulivirens* | Halmahera | Mice |
| *V. caerulivirens* | Halmahera | Mice |
| *V. caerulivirens* | Halmahera | Mice |
| *V. caerulivirens* | Halmahera | Mice |
| *V. melinus* | Sula Islands | Mice |
| *V. melinus* | Sula Islands | Mice |
| *V. melinus* | Sula Islands | Mice |
| *V. melinus* | Sula Islands | Mice |
| *V. melinus* | Sula Islands | Mice |
| *V. yuwonoi* | Halmahera | Chicken |
| *V. yuwonoi* | Halmahera | Chicken |
| *V. yuwonoi* | Halmahera | Chicken |
| *V. jobiensis* | Sorong, Papua | Chicken |
| *V. jobiensis* | Sorong, Papua | Crickets, mice |
| *V. rudicollis* | Sumatra | Crickets |
| *V. rudicollis* | Sumatra | Crickets |
| *V. panoptes* | Merauke, Papua | Crickets |
| *V. panoptes* | Merauke | Crickets, mice |
| *V. panoptes* | Merauke, Papua | Crickets, mice |
| *V. panoptes* | Merauke, Papua | Crickets, mice |
| *V. timorensis* | Roti Island | Mice |
| *V. similis* | Merauke, Papua | Crickets |
| *V. similis* | Merauke, Papua | Crickets |
| *V. similis* | Merauke, Papua | Crickets |

Figure 1. Sampling oral fluid from a *Varanus* specimen.

*Pseudomonas aeruginosa* (Poh *et al.* 1992), and *Lactococcus* (Tanskanen *et al.* 1990), (Le Bourgeois *et al.* 1989).

CHEF-DRII (BioRad Laboratories, California) was employed to separate long DNA fragments throughout the experiment. For this study, we used 0.9% agarose (Gibco BRL, New York), 2-40 s pulsed for 20 h, 0.5xTBE buffer (50 mM Tris–Boric Acid; 0.1 mM EDTA pH 8.0). The running buffer temperature was maintained at 14°C and at a constant voltage of 5.4 volts.

Analyses of banding patterns from *Spe*I schizotyping were performed using the programs of Rohlf (1990). Banding patterns were first transformed into binary data based on presence of DNA fragments on the gel (bands). The binary data matrix, obtained with the program SIMQUAL, using a single matching coefficient, was transformed into a similarity matrix. UPGMA cluster analysis was carried out employing the SAHN program (Rohlf 1990).

## RESULTS AND DISCUSSION

**Isolation and Screening of Ampicillin Resistant *E. coli*.** Based on colony morphology we obtained 13 different *E. coli* isolates from 11 *Varanus* specimens of seven different species. We could not obtain any *E. coli* from the other samples. This might have been due to too low quantities sampled from the saliva of the monitor specimens. Of the 13 isolates obtained 12 (92%) showed resistance to ampicillin (Table 2). All ampicillin resistant isolates were also tested with another antibiotic which are kanamysin, tetracycline, spectinomysine, and trimethoprim. All isolates are resistance to trimethoprim (50 µg/ml), and tetracycline (25 µg/ml). Of the 12 isolates obtained only 1 showed resistance to kanamycine (50 µg/ml) and 3 isolates showed resistance to spectinomycine (50 µg/ml). The high percentage of antibiotic, particularly ampicillin, resistance was quite unexpected. Unfortunately, we had only one sample of the yellow monitor and could not test whether the lack of ampicillin resistant *E. coli* is a general phenomenon in this species. This will be tested in further studies. The monitor specimens sampled were collected from the wild, in their natural habitats. Accordingly, the resistance we found may be "natural" resistance. For a better understanding of the possible adaptive "meaning" of the resistance patterns we require further study. Possibly, many microbial organisms may show similar resistance in their natural environments.

Table 2. Results of antibiotic-resistance tests.

| Isolate code | *Varanus* specimen | Origin | Resistance* | | | | |
|---|---|---|---|---|---|---|---|
| | | | Ap | Km | Tc | Sp | Tp |
| 2-2 | *V. prasinus* | Sorong | + | - | + | - | + |
| 4-1 | *V. salvator* | Manado | + | - | + | + | + |
| 5-1 | *V. salvator* | Manado | + | + | + | - | + |
| 6-1 | *V. salvator* | Java | + | - | + | + | + |
| 7-1 | *V. salvator* | Java | + | - | + | - | + |
| 13-1 | *V. similis* | Merauke | + | - | + | - | + |
| 16-1 | *V. indicus* | Sorong | + | - | + | - | + |
| 23-2 | *V. caerulivirens* | Halmahera | + | - | + | + | + |
| 27A | *V. melinus* | Sula Islands | - | ND | ND | ND | ND |
| 30-1 | *V. yuwonoi* | Halmahera | + | - | + | - | + |
| 30-2 | *V. yuwonoi* | Halmahera | + | - | + | - | + |
| 31-2 | *V. yuwonoi* | Halmahera | + | - | + | - | + |
| 31-4 | *V. yuwonoi* | Halmahera | + | - | + | - | + |

*Ap: Ampicillin, Km: Kanamysin, Tc: Tetracycline, Sp: Spectinomysine, Tp: Trimethoprim, ND: Not Determined.

The ampicillin resistant isolates were further characterized by a number of physiological tests using Microbact® 24 E (12E/12A+12B). *E. coli* isolates were similar in 22 of the 26 parameters analyzed. Differences in the ability to utilize the carbon sources sorbitol, sucrose, raffinose, and arginine could be assigned to 7 different physiological groups (A-G; Table 3). In terms of species, group A consisted of *V. indicus*, *V prasinus*, *V salvator*, *V. similis*, and *V. yuwonoi*. Group B and C comprised only *V. salvator*, groups D, E, and F only *V. yuwonoi*, and group G only *V. caerulivirens*. Accordingly, there is neither a clear grouping with regard to species nor with regard to their localities. This may indicate that physiological characteristics exhibited high levels of individual variation, which were influenced by envitonmental factors. Therefore, physiological characteristics in this study revealed unsuitable to evaluate taxonomic relationships among the species sampled. Another possibility of explaining the antibiotic resistance we found in the monitors would have been intake of the relevant bacteria with the food. Our results, however, indicate that the different physiological types of *E. coli* were independent of the kind of food administered to the specimens. For instance, specimens that were exclusively fed with chicken showed differences in the physiological characters studied (Table 4).

Table 3. Physiological characteristics of *E.coli* employing Microbact® 24E (12E/12A + 12B).

| Substrate | Isolate | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2-2 | 4-1 | 5-1 | 6-1 | 7-1 | 13-1 | 16-1 | 23-2 | 30-1 | 30-2 | 31-2 | 31-4 |
| Oxidase | - | - | - | - | - | - | - | - | - | - | - | - |
| Motility | + | + | + | + | + | + | + | + | + | + | + | + |
| Lysine | + | + | + | + | + | + | + | + | + | + | + | + |
| Ornithine | + | + | + | + | + | + | + | + | + | + | + | + |
| $H_2S$ | - | - | - | - | - | - | - | - | - | - | - | - |
| Glucose | + | + | + | + | + | + | + | + | + | + | + | + |
| Manitole | + | + | + | + | + | + | + | + | + | + | + | + |
| Xylose | + | + | + | + | + | + | + | + | + | + | + | + |
| ONPG | + | + | + | + | + | + | + | + | + | + | + | + |
| Indole | + | + | + | + | + | + | + | + | + | + | + | + |
| Urease | - | - | - | - | - | - | - | - | - | - | - | - |
| V.P. | - | - | - | - | - | - | - | - | - | - | - | - |
| Citrate | - | - | - | - | - | - | - | - | - | - | - | - |
| TDA | - | - | - | - | - | - | - | - | - | - | - | - |
| Gelatine | - | - | - | - | - | - | - | - | - | - | - | - |
| Malonate | - | - | - | - | - | - | - | - | - | - | - | - |
| Inositol | - | - | - | - | - | - | - | - | - | - | - | - |
| Sorbitol | + | + | + | + | + | + | + | + | + | + | - | - |
| Rhamnose | + | + | + | + | + | + | + | + | + | + | + | + |
| Sucrose | - | - | - | - | - | - | - | + | - | - | - | - |
| Lactose | + | + | + | + | + | + | + | + | + | + | + | + |
| Arabinose | + | + | + | + | + | + | + | + | + | + | + | + |
| Adonitol | - | - | - | - | - | - | - | - | - | - | - | - |
| Raffinose | + | + | + | + | - | + | + | + | + | - | + | + |
| Salicine | - | - | - | - | - | - | - | - | - | - | - | - |
| Arginine | - | - | + | + | - | - | - | - | - | + | + | - |
| Character | A | A | B | B | C | A | A | G | A | D | E | F |

Tabel 4. Isolate relationships between *Varanus* specimens, their origin, feed, physiological characteristics, and schizotype patterns.

| Isolate Code | *Varanus* specimens | Origin | Feed | Physiological Characteristic | Schizotype Patterns |
|---|---|---|---|---|---|
| 2-2 | *V. prasinus* | Biak | Mice | A | Smeared |
| 4-1 | *V. salvator* | Manado | Chicken | A | 1 |
| 5-1 | *V. salvator* | Manado | Chicken | B | 2 |
| 6-1 | *V. salvator* | Java | Chicken, mice | B | 3 |
| 7-1 | *V. salvator* | Java | Chicken, mice | C | 4 |
| 13-1 | *V. similis* | Merauke | Chicken, mice | A | Smeared |
| 16-1 | *V. indicus* | Sorong | Chicken, fish, mice | A | Smeared |
| 23-2 | *V. caerulivirens* | Halmahera | Mice | G | 3 |
| 30-1 | *V. yuwonoi* | Halmahera | Chicken | A | 4 |
| 30-2 | *V. yuwonoi* | Halmahera | Chicken | D | 5 |
| 31-2 | *V. yuwonoi* | Halmahera | Chicken | E | 6 |
| 31-4 | *V. yuwonoi* | Halmahera | Chicken | F | 6 |

Antibiotic resistance patterns and physiological characters seem to be suitable for diversity analyses among *E. coli* isolates. Nevertheless, both antibiotic resistance and physiological characters are phenotypic characters, which may be very influenced by environmental factors. To better understand about diversity in *E. coli* we used genetic diversity employing pulsed filed gel electrophoresis (PFGE). PFGE is the most discriminative method for genetic analyses in species level. Poh *et al.* (1992) reported that DNA profiling employing PFGE is more discriminative than ribotyping.

**Genetic Diversity of *E. coli* Isolates.** DNA profiling employing PFGE yielded discrete and evenly distributed DNA binding patterns for nine isolates (Figure 2), while it produced smeared DNA for the other three isolates (isolates 2-2, 5-1, and 16-1, respectively). The degraded or smeared DNA was probably caused by a specific endonuclease that could not be inactivated during the gel insert preparation for isolation of intact genomic DNA (Lai *et al.* 1989).

Based on the results of genetic analyses, five of the nine isolates were genetically unique and might reflect natural association between specific *E. coli* strains and a particular individual monitor lizard. Each of the *E. coli* isolates might belong to the normal oral microbiota of each monitor specimen. The DNA profile of isolate 31-2 had a banding pattern identical to that of isolate 31-4. Both isolates were from the same monitor lizard. Even though *E. coli* strains may be identical, these may show different physiological characteristics. Possibly different conditions in the microenvironment of the buccal affect gene expression in *E. coli* isolates. For instance, isolates 30-1 and 30-2 were also from the same specimen, but they showed differences both in DNA profiles and physiological characteristics. It might be possible that in the same environment or microenvironment two or more bacterial strains could occur sympatrically. The results of our genetic analyses also showed that isolate 6-1 had a DNA profile identical with that of isolate 23-2, whereas both of them had different physiological characteristics. Isolates 6-1 and 23-2 were from different *Varanus* species which both had been fed with mice. This may indicate cross contamination from the feed. If this applies, then gene expression of *E. coli* strains may have been influenced by the microenvironmental conditions in the buccal of the lizards.

UPGMA cluster analysis of *Spe*I schizotypes revealed three main clusters of *E. coli* strains (Figure 3). These were (1) the cluster consisting of isolates 4-1, 5-1, and 7-1, (2) the cluster of isolates 30-1, 30-2, 31-4 and 31-2, and (3) the cluster of isolates 23-2 and 6-1. In general, these results indicated that there was a relationship between the genetics of the isolates and the origin of the respective monitor specimens. In case of isolate 23-2, which was isolated from *Varanus caerulivirens*, we obtained close similarity to the schizotypes of isolate 6-1, which was isolated from *V. salvator*. Both isolates, however, were different in physiological characteristics. This difference might have been due to plasmid content or smaller site DNA finger printings (< 50 kb).

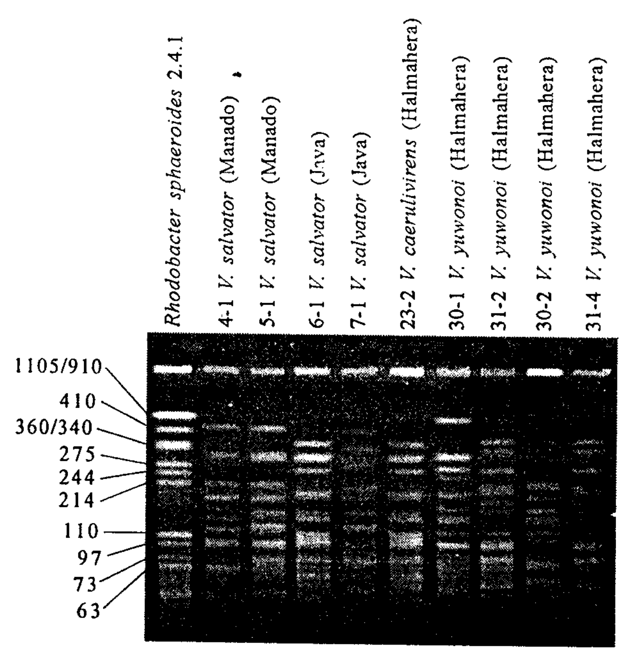


Figure 2. Profile of *Spe*I-schizotyping of *E. coli* isolates isolated from the buccal of Indonesian monitor lizards (*Varanus* spp.). Running condition: 0.9% high melting point agar, 0.5 x TBE buffer, buffer temperature 14°C, ramping pulse time 2-40 seconds, running time 20h, voltage 5.4 volt/cm² (18-21 mA).

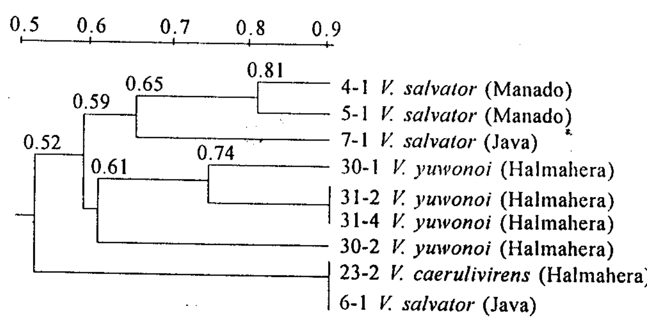


Figure 3. Dendrogram derived from *Spe*I-schizotyping of *E. coli* isolates.

Our study has shown that the buccal environment of monitor lizard may contain highly variable bacterial strains which even show resistance to antibiotics. Further studies may clarify whether the microbial composition of the mouth cavity of monitor lizards is species-specific or specific for particular environmental conditions. Our present work on the resistance genes may reveal new genes, which code for resistance against certain antibiotics. Moreover, our approach might even shed new light on the phylogenetic relationships amongs varanid lizards.


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