Vet.Med.J.,Giza. Vol.49, No.1. (2001):111-121.

EXOTOXIGENICITY OF AEROMONAS SPECIES ISOLATED FROM DIFFERENT SOURCES : INHIBITORY EFFECT OF ESSENTIAL OILS ON THEIR GROWTH

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Received: 13. 11. 2000
Accepted: 20. 12. 2000

SUMMARY

A total of 74 water samples (35 canal water and 39 chlorinated tap water), 49 feed samples (22 meat meal and 27 fish meal) and 49 faecal samples from diarrhoeic animals (24 from sheep and 25 from goats) were examined for *Aeromonas* species. The highest incidence of isolation was found in fresh canal water samples 40%, (14 out of 35 samples) and in chlorinated water samples 23.1% (9 out of 39 samples). The isolation of *Aeromonas* species from feed samples (meat and fish meals) was more or less similar (22.85% and 22.0% respectively). Faeces from diarrhoeic sheep and goats revealed the isolation of *Aeromonas* species in 16.7% and 20% respectively. *Aeromonas* hydrophila was most dominant and was isolated from all different samples 65.1% of all isolates (28 out of 43 isolates). Whereas *Aeromonas sobria* and *Aeromonas caviae* were isolated in much lower rates 20.1% (9 isolates) and 14% (6 isolates) respectively. Growth at 43°C, haemolysin production, enterotoxigenicity as well as cytotoxigenicity were expressed by *A. hydrophila* and *A. sobria* more significantly ($P<0.05$) than *A. caviae*. Most of the *A. hydrophila* and *A. sobria* isolates were enterotoxigenic (82.1% and 88.9% respectively) and could grow at 43°C and produce high haemolysin titers (1/128-1/1024). Essential oils (clove coriander and peppermint) induced inhibitory effect on the growth of *Aeromonas* isolates. Clove oil had MIC of 500µg/ml, whereas coriander and peppermint oils had 1,250µg/ml and 12,500µg/ml respectively. There was no difference in the antibiogram of *A. hydrophila, A . sobria* and *A. caviae*. Most strains were found sensitive to gentamicin, kanamycin and chloramphenicol and resistant to penicillin, ampicillin, cephalosporine, novobiocin and bacitracin.

INTRODUCTION

Aeromonas species are ubiquitous Gram negative

psychrotrophic bacteria , that can grow at 4°C. In recent years these bacteria have been increasingly recognized as enteric pathogen for human and animals. The bacteria have been also implicated in a diverse pathogenic conditions ranging from gastroenteritis, peritonitis, cellulitis, osteomylitis, meningitis and septicaemia. *Aeromonas* have been also considered as a major water borne microorganisms that causes the infection as a result of the exposure to contaminated water (Daily et al.1981, El Shenawy and Marth 1990, Efuntoye 1995 and Paniagua et al 1998). As *Aeromonas* species are widely disseminated in diverse ecological niches e.g. in water, soil, foods of animal origin (milk, eggs, meat), and vegetables, they constitute a hazardous source of infection to the immuno-compromised patients as those with HIV as well as to animals under stress or in poor hygienic conditions(Gracey et al. 1982, Krovacek et al. 1989, Stecchini et al 1993).

Many putative virulence factors, that commonly exist in Gram negative bacteria, have been associated with *Aeromonas* species, including heat stable enterotoxins, adhesins, outer membrane proteins, heat labile cytotoxins, haemolysins, proteases, phospholipases, nucleases, elastases, amylase and lecithinase. Some of these virulence potentials as cytotoxigenicity have been correlated to clinical isolates of *A. hydrophila* or *A. sobria*, however, the investigation of enterotoxigenicity of *Aeromonas* in rabbit ileal loop system varied with the source of isolate (Davis et al. 1978, Daily et al.1981, El Shenawy and Marth 1990, Wadstrom and Ljungh 1991, Seleim 1996).

The purpose of this study was to isolate the *Aeromonas* species from different niches (canal water, chlorinated tap water, animal feed as meat meal and fish meal as well as faeces from diarrhoeic sheep and goats) and identify some of the virulence factors associated. Also the effect of some essential oils as well as antimicrobial agent on the growth of these isolates was evaluated.

MATERIALS AND METHODS

Samples

A total of 74 water samples (35 canal water and 39 chlorinated tap water), 49 feed samples (22 meat meal and 27 fish meal) and 49 faecal samples from diarrhoeic animals (24 from sheep and 25 from goats) were collected from governmental and private farm premises in Giza and Dakahlia Governorates. Water was collected in sterile glass bottles from the drinking droughts or canals nearby the farms. Faecal samples were collected in sterile plastic bags from diarroeic sheep and goats aged 1-3 years. All samples were transported to the laboratory in ice box and examined for *Aeromonas* species.

Vero cell cultures were kindly provided from the Virology Department, Animal Health Research Institute, Dokki, Cairo.

Isolation and Identification of Aeromonas species :

a) **water samples:** 100ml quantity of water sample was filtered through 0.45µm pore size membrane filter (Schleicher and Schull, Germany). The membrane was then transferred to Lauryl sulphate agar plate and incubated over night at 37°Cfor 24hrs.

b) **Feed samples:** cultured indirectly into brain heart infusion broth (BHI Oxoid), then transferred to Lauryl sulphate agar plate and incubated over night at 37°Cfor 24hrs.

c) **Faecal samples:** cultured directly onto Lauryl sulphate agar plate and incubated over night at 37°Cfor 24hrs.

Isolated colonies were subcultured onto sheep blood agar and tested for Gram staining, oxidation and fermentation of glucose, cytochrome oxidase, fermentation of glucose, manitol, sucrose, arabinose, esculine hydrolysis, gas from glucose, growth on KCN, B-galactosidase, arginine dihydrolase, indole production, ornithine decarboxylase, tryptophane deaminase, urease, production of H_2S, fermentation of inositol, sorbitol, rhamnose, melibiose, lysine decarboxylase, growth on citrate, Voges Proskauer, nitrate reduction, and resistance to 2,4 diamino 6,7 diisopropylpteridine (O/129). Isolated colonies were also identified using the API 20E system (Analytab products, USA) and were read after 18-24hrs at 37°C. (Daily et al. 1981, Kirov et al. 1986, Araujo et al. 1991).

Growth at 43°C

Isolates were tested for its ability to grow onto blood agar plates at 43°C for 24hrs. growth was scored from 0 (no growth) to 4+ (heavy growth) (Kirov et al. 1986).

Enterotoxin assay

Aeromonas isolates from 18-24hrs blood agar cultures were inoculated into 5ml tryptone soy broth (Difco) supplemented with 0.6% yeast extract, then incubated at 37°C for 24hrs with agitation at 200 to 300rpm. Cell free supernatant was then prepared by centrifugation at 10,000xg for 30min and filtration by 0.2µm membrane filter. The supernatant was tested at the day of preparation by injecting 0.1ml of culture filtrate through the abdominal wall into the milk filled stomach of mice 2-4 days old. Other mice were injected with 0.1ml saline as negative control the mice were kept for 3 hrs, then were killed . The intestines were removed and the ratio of the intestines weight to the remaining body weight was determined. A ratio greater than 0.083 was recorded as positive test for enterotoxins. (Kirov et al 1986, Robins-Brown et al. 1993).

Cytotoxin assay

The *Aeromonas* isolates were grown into BHI broth at 37°C for 18-24hrs on a rotator shaker at 250rpm and were inoculated onto Vero cells, which were examined microscopically for cytotoxic effects. Isolates that produced shrinking vacuolization and crenation at titers of >1:8 was considered positive for cytotoxine activity (Daily et al. 1981, Giugliano et al. 1982).

Hemolysin assay

Double dilution of the cell free supernatant of *Aeromonas* isolates in phosphate buffer saline pH7.4 were made in microtiter plate (Nunc Co.) and an equal volume of 100µl of 1% fresh washed rabbit erythrocytes was added. Phosphate buffer saline and broth blanks were included in each plate. Each isolate was assayed in duplicate. Plates were sealed and incubated at 37°C for 1hr then at 4°C for another 1hr. The hemolysin titer was determined as the last dilution showing 50% hemolysis of the erythrocytes (Daily et al 1981, Kirov et al. 1986).

Determination of the minimum inhibitory concentration (MIC) of essential oils

The minimum inhibitory concentration of the clove, coriander and peppermint oils were determined by adding different oil concentrations 250, 500, 750, 1000, 1250, 1500µg/ml clove and coriander or 5, 7.5, 10, 12.5 and 15mg/ml peppermint oil to 10ml 1.2% tryptic soy agar (TSA), tempered at 45°C. The oil was thoroughly mixed for 10s before pouring the plates.0.1ml ethanol with no essential oils were added to TSA and served as control. Loop aliquots of *Aeromonas* isolates 10^7 and 10^4CFU/spot in BHI (18hrs at 30°C) were used to spot TSA plates. Plates were incubated at 30°C for 24-48hrs, and the MIC was determined for each oil as the lowest concentration of the oil at which no colonies appear on the TSA plates (Stecchini et al. 1993).

Antimicrobial sensitivity testing

Aeromonas isolates were tested for their sensitivity to different antimicrobial agents Gentamicin 10µg, Kanamycin 30µg, Chloramphenicol 30µg, Tetracycline 30µg Neomycin30µg, Streptomycin 10µg Trimethoprim sulphamethazole 25µg, Penicillin 10units, Ampicillin 10µg, Cephalosporine 30µg, Novobiocin 30µg and Bacitracin 10µg was tested by the agar disc diffusion method (Bauer et al 1966).

RESULTS

The results of the isolation of *Aeromonas* species from different sources, showed that the highest incidence of isolation was in fresh canal water samples 40%, (14 out of 35 samples) followed by

chlorinated water samples 23.1% (9 out of 39 samples). The isolation of *Aeromonas* species from feed samples, meat and fish meals was more or less similar (22.85 and 22.0% respectively). Faeces from diarroeic sheep and goats revealed the isolation of *Aeromonas* species in 16.7% and 20% respectively.

A. hydrophila was the most dominant species isolated from the different kinds of samples 65.1% (28 out of 43 isolates), whereas *A. sobria* and *A. caviae* were isolated in much lower rates 20.1% (9 isolates) and 14% (6 isolates) respectively (Table1).

The biochemical profile of all the *Aeromonas* isolates were uniformly positive for cytochrome oxidase, fermentation of glucose, manitol, sucrose,B-galactosidase,arginine dihydrolase and indole production, and were uniformily negative for ornithine decarboxylase, tryptophane deaminase,urease, production of H_2S, and fermentation of inositol, sorbitol, rhamnose and melibiose. The tests that produced variable results were, lysine decarboxylase, growth in citrates, , Voges Proscauer, ,nitrate reduction, arabinose fermentation, esculine hydrolysis, gas from glucose, growth on KCN and resistance to 2,4diamino

(Table 1) Prevalence Of Aeromonas Species Isolated From Different Sources

Isolate	Water		Feed		Faeces .		Total
	Fresh Canal water n=35	Chlorinated water n=39	Meat meal n=22	Fish meal n=27	Sheep n=24	Goats n=25	n=172
A. hydrophila	9	6	3	4	3	3	28
A. sobria	3	2	1	1	1	1	9
A. caviae	2	1	1	1	0	1	6
Total	14 (40%)	9 (23.1%)	5 (22.8%)	6 (22.2%)	4 (16.7%)	5 (20%)	43 (25%)

n= number of samples tested

(Table 2) Biochemical Profile Of *Aeromonas* Species Isolated From Different Sources

Biochemical test	*A. Hydrophila* No. positive/total	*A. Sobria* No. positive/total	*A. caviae* No. Positive/total
Lysine decarboxylase	16/28	5/9	2/6
Growth in citrate	4/28	4/9	1/6
Voges Proskauer	28/28	9/9	0/6
Arabinose fermentation	28/28	0/9	6/6
Esculine hydrolysis	28/28	0/9	6/6
Gas from glucose	28/28	9/9	0/6
Nitrate reduction	18/28	6/9	3/6
Growth in KCN	26/28	5/9	2/6
Resistance to O/129	26/28	4/9	3/6

(Table 3) Virulence Characteristics Of *Aeromonas* Species

Isolate	Growth at 43°C	Haemolysin	Enterotoxin	Cytotoxin
A. hydrophila	24/28*	23/28	23/28	20/28
A. sobria	8/9	7/9	8/9	5/9
A. caviae	3/6	2/6	2/6	1/6
Total	35/43 81.4%	32/43 74.4%	33/43 76.7%	26/43 60.5%

* No. positive / total.

(Table 4) Haemolysin Titer Of Different *Aeromonas* Species

Isolate	Haemolysin titer					
	1/32	1/64	1/128	1/256	1/521	1/1024
A. hydrophila	2/28*	3/28	8/28	9/28	4/28	2/28
A. sobria	1/9	1/9	2/9	2/9	2/9	1/9
A. caviae	2/6	2/6	1/6	1/6	0/6	0/6

Haemolysin titration <1/128 is considered positive
* No. positive / total.

(Table 5) Inhibitory Effect Of Essential Oils On *Aeromonas* Species

Isolate	Olove oil			Coriander oil			Peppermint oil		
	250µg/ml	500µg/ml	1000µg/ml	1250µg/ml	1500µg/ml	5000µg/ml	10,00µg/ml	12500µg/ml	15000µg/ml
A. hydrophila	28/28*	0/28	0/28	28/28	0/28	0/28	28/28	0/28	0/28
A. sobria	9/9	0/9	0/9	9/9	0/9	0/9	9/9	0/9	0/9
A. caviae	6/6	0/6	0/6	6/6	0/6	0/6	6/6	0/6	0/6

* No. of positive growth / total.

(Table 6) Antibiogram of the different *Aeromonasn* species.

Chemotherapeutic agents	Concentration.	*A. hydrophila*	*A. sobria*	*A. caviae*
	µg	Sensitive (%)	Sensitive (%)	Sensitive (%)
Gentamicin	10	28/28* 100%	9/9 100%	6/6 100%
Kanamycin	30	27/28 96.4%	8/9 88.9%	6/6 100%
Chloramphenicol	30	27/28 96.4%	9/9 100%	6/6 100%
Tetracycline	30	26/28 92.9%	7/9 77.8%	5/6 83.3%
Neomycin	30	20/28 71.4%	5/9 55.6%	3/6 50%
Streptomycin	10	17.28 60.7%	6/9 66.7%	3/6 50%
Trimethoprim sulphamethazole	25	10/28 35.7%	4/9 44.4%	2/6 33.3%
Penicillin	10 units	0/28 0%	0/9 0%	0/6 0%
Ampicillin	10	0/28 0%	0/9 0%	0/6 0%
Cephalosporine	30	2/28 7.1%	1/9 11.1%	1/6 16.7%
Novobiocin	30	1/28 3.6%	2/9 22.2%	1/6 16.7%
Bacitracin	10	2/28 7.1%	1/9 11.1%	2/6 33.3%

* No positive/total

6,7diisopropylpteridine (O/129) (Table 2).

Virulence characteristics of the *Aeromonas* isolates varied greatly. *A. hydrophila* isolates as well as *A. sobria* showed a higher expression of virulence factors than *A. caviae* (Table 3). Most of the *A. hydrophila* and *A. sobria* isolates that were enterotoxigenic could grow at 43°C and were found to produce high haemolysine titers (1/128-1/1024). Whereas the non enterotoxigenic were unable to grow at 43°C and non haemolysine producing. Also it was found that the majority of cytotoxic producing isolates were also positive for enterotoxin production.

The use of essential oils induced inhibitory results on the growth of *Aeromonas* isolates. There was no discrepancies in the effect of the essential oils on inhibition pattern of the three *Aeromonas* species. Clove oil had MIC 500µg/ml, whereas coriander and peppermint oil had 1,250 and 12,500µg/ml respectively (Table 5). There was no difference in the antibiogram between the three *Aeromonas* species. Most strains were found sensitive to gentamycin, kanamycin and chloramphenicol and resistant to penicillin ampicillin, cephalosporine, novobiocin and bacitracine.

DISCUSSION

A.hydrophila, A. sobria and *A. caviae* has been increasingly incriminated in causing a wide range of illnesses in human and animals. Previous reports confirmed the isolation of these bacteria from fresh and chlorinated water. Drinking water contaminated with *Aeromonas* species can even cause abortion in buffaloes and cows (Das and Paranjape 1990, Das 1991). Many authors reported about out-breaks of watery diarrhoea containing mucus in sheep, goats, pigs and rabbits and *Aeromonas* species had been isolated in pure cultures from them. The organism was also isolated from the beef, lamb, pork as well as the milk of the lactating animals (Kirov et al.1986, Drew and greenway 1990, Araujo et al. 1991, Ibraham and Mac Rae 1991, Efuntoye 1995, Rajesh et al. 1997 Sing et al. 1997). In this study *Aeromonas* species were isolated from different sources (fresh canal water and chlorinated water, as well as from feed samples). This could explain the isolation of the organisms from the faeces of diarrhoeic sheep and goats which consume contaminated water and feed staff. The highest incidence of isolation was from canal water samples 40%, (14 out of 35 samples) and chlorinated water samples 23.1% (9 out of 39 samples) which also agreed with Araujo et al. (1991).

The isolation of Aeromonas from meat meals and fish meals were almost similar (22.85% and 22.0% respectively) which could be attributed to contamination of the raw meat used for the manu-

facturing of these meals or due to post-manufacturing contamination.

Many putative virulence factors, have been associated with Aeromonas species, including heat stable enterotoxins, adhesines, outer membrane proteins, heat labile cytotoxins, haemolysins, proteases, phospholipases, nucleases, elastases, amylase and lecithinase. Some of these virulence potentials as cytotoxigenicity have been correlated to clinical isolates only (Lund et al 1991, Wadstrom and Ljungh 1991 Thornton et al. 1993, Angka et al. 1995, Agarwal et al. 1998, Ling et al. 1999). The results showed that *A. hydrophila* as well as *A. sobria* had significantly higher ($P< 0.05$) virulence characteristics than *A. caviae* (Table 3), which supported the previous findings of Daily et al. 1981 and Kirov et al.1986. Moreover most of the *A. hydrophila* and *A. sobria* isolates that were enterotoxigenic, could grow at 43°C and to produce high haemolysin titers (1/128-1/1024) (Table 4). Whereas the non enterotoxigenic were unable to grow at 43°C and did not produce haemolysin. Also it was noticed that the majority of cytotoxic producing isolates were also positive for enterotoxin production These findings also were found to agree with. Daily et al. 1981 Kirov et al. 1986 and Araujo et al. 1991.

There was no difference in the antibiogram between the three *Aeromonas* species. Most strains were found sensitive to gentamicin, kanamycin and chloramphenicol and resistant to penicillin, ampicillin, cephalosporine, novobiocin and bacitracin. These results agreed with Wang et al., 1999. Many authors ruled out the use of antibiotics and favored the use of essential oils as clove, coriander and peppermint that exhibited antimicrobial activity against wide range of Gram positive and Gram negative bacteria at a very low concentrations without mutagenicity (Deans et al. 1995, Kono et al. 1995, Direkbusarakom et al 1997 and Montes et al. 1998). The results of this study revealed that, the essential oils, clove, coriander and peppermint had inhibitory effect on the three *Aeromonas* species. Clove had MIC 500µg/ml, whereas coriander and peppermint had 1,250 and 12,500µg/ml respectively (Table 5). These results encourage the use of essential oils as substitute for antibiotics, and can be added to the animal feeds in low concentrations without any harmful side effect (Kono et al. 1995, and Montes et al. 1998).

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