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ABSTRACT

The survival of four biotypes of *Yersinia enterocolitica* (2 isolated from swine and 2 from *Chironomus* larvae) has been assessed in two types of surface water, with low and high content of organic matter, at 3 different temperatures. The results have shown that the bacteria undergo active replication during the first weeks of incubation; thereafter, they can survive up to 74 weeks, with the survival time strongly influenced by the incubation temperature. Therefore, surface waters may be an important reservoir for the biotypes considered and thus, they may play a strategic role in the epidemiology of *Yersinia* infections.

Indexing terms/Keywords

Yersinia enterocolitca; *wate;, survival*

Academic Discipline And Sub-Disciplines

Microbiology

SUBJECT CLASSIFICATION

Epidemiology

TYPE (METHOD/APPROACH)

Study of *Yersinia enterocolitica* survival in aquatic environment

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INTRODUCTION

Yersinia enterocolitica is responsible for human enteric syndromes spread all over the world and has also been isolated from both healthy and sick animals (cattle, pigs, sheep, dogs, cats, birds, etc.). The main sources of infection for humans consist of meat, milk, vegetables and water contaminated by faeces of human and animal origin (1). Although pigs have been found to be a major reservoir of human pathogenic strains (2), it is only more recently that the organism has become recognized for its waterborne transmission potential and hence referred to as an emerging waterborne pathogen (3, 4).

Yersinia enterocolitica is potentially a pathogenic bacterium transmitted through the faecal-oral route. Illness caused by *Y. enterocolitica* infection is referred to as yersiniosis and there are a wide variety of disease outcomes that can result. Typical disease symptoms include those associated with gastrointestinal disease, such as fever, abdominal pain and diarrhoea (1, 5). However, the consequences of infection can be very serious, particularly in sensitive populations like the young, the elderly, subjects with chronic disease and immunocompromised ones (4, 6). Infection causes a wide range of clinical symptoms depending on factors such as the patient's age and health, as well as the serotype of the strain. Symptoms range from mild self-limiting diarrhoea (gastroenteritis) to inflammation of the small intestine (acute terminal ileitis) or inflammation of the mesenteric lymph nodes (acute mesenteric lymphadenitis) that can lead to pseudoappendicitis (5, 6). Infants and young children usually acquire gastroenteritis, while older children and young adults are more likely to develope acute terminal ileitis (5) or pseudoappendicitis (2). Post infection manifestations sometimes occur in adults, including reactive arthritis, inflammation of fat cells under the skin (erythema nodosum), inflammation of blood vessels in the kidneys (glomerulonephritis) and inflammation of the muscular tissue in the heart (myocarditis) (2, 5).

Although rare, certain patients may be predisposed to severe complications like septicaemia (occurring when bacteria get into the bloodstream) (2), which can then lead to other serious developments (5). Patients susceptible to septicemia include the young, the elderly and the immunocompromised, in particular patients suffering from diseases associated with iron overload, cancer, liver disease and patients on steroid therapy (6).

Previous surveys have detected *Y. enterocolitica* in surface water in various parts of the world, and studies have found drinking untreated water to be a possible risk factor for *Y. enterocolitica* infection. Pathogenic *Y. enterocolitica* have been implicated in a few cases of waterborne illness in humans (7, 8, 9, 10, 11, 12) and case-controlled studies have identified drinking untreated water as a risk factor for yersiniosis (13, 14).

Given that *Y. enterocolitica* is associated with animal hosts and shed in the faeces of infected animals, it is reasonable to assume that waterborne transmission of *Y. enterocolitica* may be occurring, similar to other agriculturally important microbial pathogens. Nonetheless, there have been few studies reporting on the prevalence of pathogenic *Y. enterocolitica* strains in water and none that have enumerated *Y. enterocolitica* in water.

Although *Y. enterocolitica* thrives in the intestines of warm-blooded animals, it also survives very cold temperatures and is considered psychotrophic, unlike other members of the family *Enterobacteriaceae* (15), which includes many enteric pathogens.

In preliminary tests, Harvey *et al.* (1976) (16) demonstrated that *Y. enterocolitica* could survive in refrigerated water for 6 months. Another study found that the viable cell count for *Y. enterocolitica* increased over the first 72 hours of incubation in sterile distilled water at temperatures of 4, 25 and 37°C (9). After 72 hours, viable cell counts leveled off but did not decrease, indicating the cells continued to survive for 216 hours.

Another investigation followed the survival of several different bacterial pathogens in sterile distilled water over the course of many years (17). Bacteria studied were strains isolated from fruits and vegetables. Experiments were conducted at room temperature in the dark. Two *Y. enterocolitica* strains were tested and both were found to survive for at least 5 years.

Since the results of the above researches have appeared contradictory and not easily comparable, it seemed interesting to investigate four biotypes of *Y. enterocolitica*, isolated from different sources, inoculated into two types of surface waters with different chemical and physical properties and incubated at three different temperatures, in order to plot a curve of survival in relation to each variable considered.

MATERIALS AND METHODS

The strains under investigation were isolated in previous studies and typed as 1A (strain 3Y1), 2/O9/X3 (strain A), 3 (strain 20y28) and 4/O3/VIII (strain S44). The strains 1A and S44 were isolated from the palatine tonsils of regularly slaughtered pigs and typed by serotype and phage type at the National Reference Center for *Yersinia*, University "La Sapienza" of Rome (Italy); strains 3Y1 and 20y28 were isolated from frozen feed (*Chironomus*), utilized for ornamental fish. The two types of water in which was followed the survival of the strains were taken from the Parma stream in the month of January 2013 (water 1) and from the Naviglio waterway (water 2), in both cases in the stretch immediately downstream of Parma city (Italy); the samples were subjected to chemical-physical analysis, in order to determine the basic parameters.

Incubation temperatures were equal to 5°C, 15°C and 25°C.

The survival control is continued, with cadence weekly initially, until disappearance of viable cells as regards the incubation at 15°C and 25°C, and was stopped after 74 weeks relatively to incubation at 5°C.

The strains, lyophilized, have been reborn with saline solution and cultured for three times on TSA (Tryptone Soya Agar, Oxoid) in order to revitalize the cells.

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The two samples of water, distributed in 12 flasks of 250 ml at a rate of 100 ml each, were sterilized by autoclaving at 121°C for 20 min. and stored in the dark at temperatures of 5, 15 and 25°C.

The strains, coming from broth-cultures in TBS (Tryptone Soya Broth, Oxoid) for 24 hours at 25°C, were subjected to appropriate dilutions in saline solution to obtain a final concentration of 10^7 cells/ml, as assessed by spectrophotometer reading and comparison with the scale of McFarland.

Each flask was seeded with 1 ml of the bacterial suspension so as to obtain a final concentration of 10^5 cells/ml, controlled by plate counts with incubation at a temperature of 25°C.

On a weekly basis, for the first 18 weeks, and then biweekly, counts on TSA plates were performed in duplicate, always with incubation at 25°C, in order to ascertain the number of viable cells. The validity of the calculations for each pair of plates was assessed by calculating the χ^2 (18).

RESULTS

The chemical-physical parameters of the two water samples are listed in Table 1: all values, with the exception of pH, were significantly higher in water 2 than in water 1.

Table 1: Physical and chemical characteristics of the two water samples and their origin

Parameters	Water 1	Water 2
pH	7.75	7.77
Hardness °F	11	22
M-NH_4	0.14	0.35
M-NO_2	0.02	0.09
P-PO_4	0.07	0.11
COD	5.6	10.4
Origin	Parma stream	Naviglio Waterway

°F: French Degreed

COD: chemical oxygen demand

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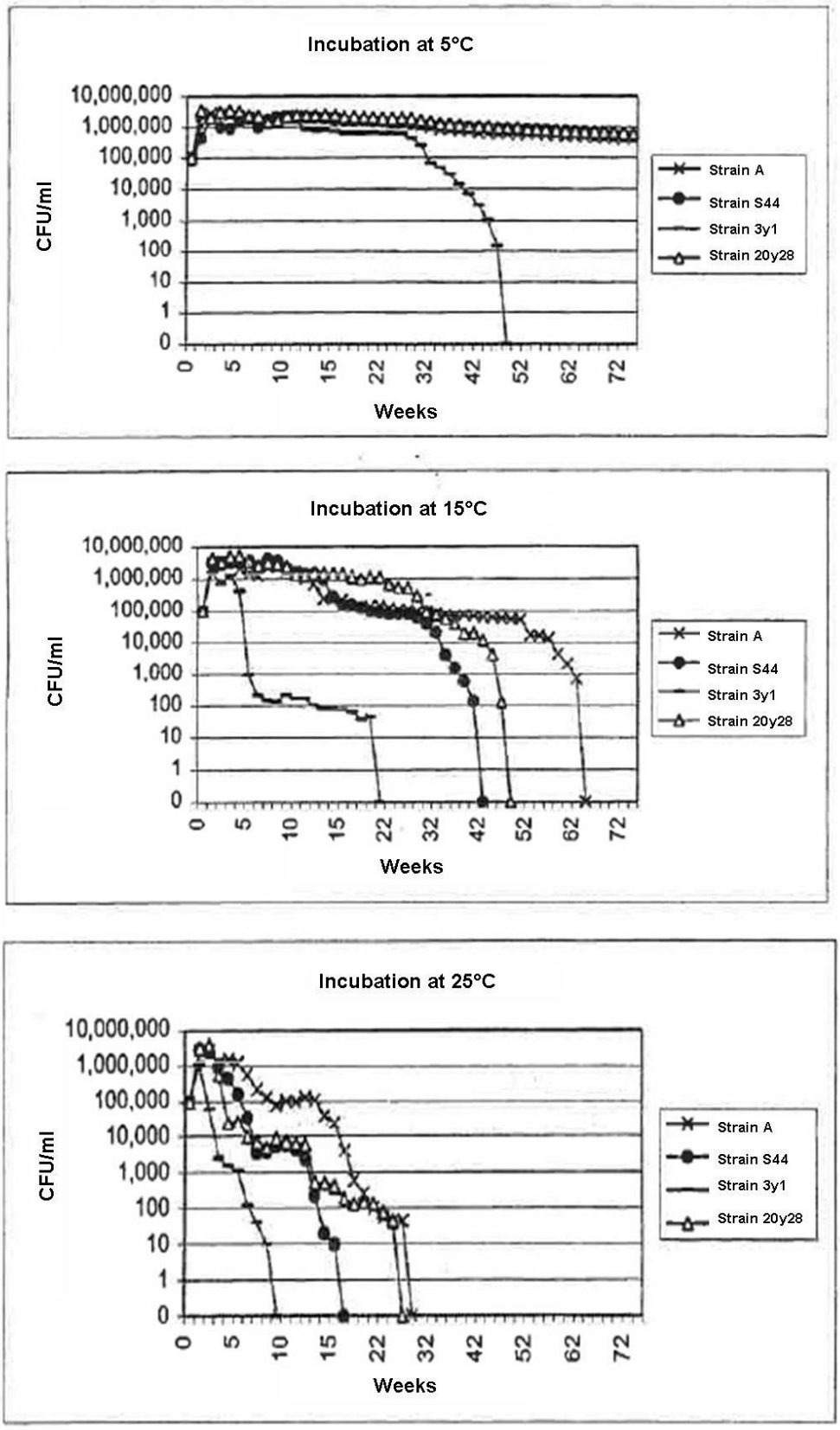

Figure 1: Water 1 - Survival curves of strains in relation to incubation temperature

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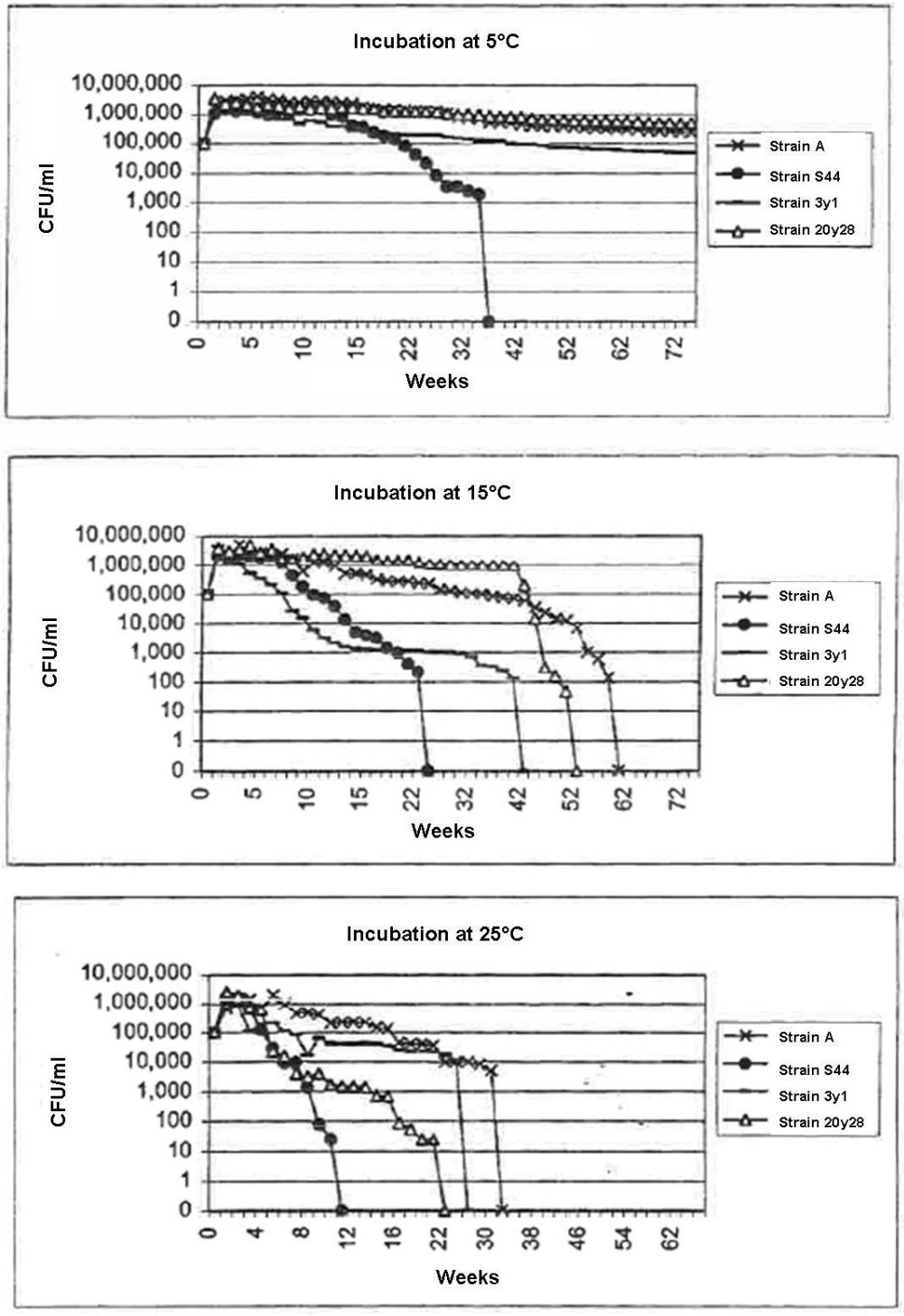

Figure 2: Water 2 - Survival curves of strains in relation to incubation temperature

Figures 1 and 2 summarize in graphical form the results concerning the survival curves of the four strains in the two water samples, in relation to the three incubation temperatures.

The initial assessment makes clear that in the two initial weeks of incubation the number of viable cells increases, regardless of the variables water-temperature, from a minimum of 1 to a maximum di 1,6 logarithms.

From the third week, the influence of incubation temperature appears and the behavior of the strains is affected, with a deep decrease at 25°C, rapid rising at 15°C - which allowed count up to 5,300,000 cells/ml - and a substantial stability at 5°C.

In principle, regardless of the type of water, the longevity of the strains is correlated to the temperature level, in the sense that at 25°C the survival has not continued beyond the 35th week, at 15°C over the 64th, while at 5°C all strains, except for two (3Y1 in the water 1 and S44 in the water 2) showed counts very high, generally well above of the *inoculum*, even after 74 weeks of incubation.

Most absolute counts was observed at 15°C with a concentration in cells/ml equal to 4,900,000 for the strain A in the 3rd week, 3,700,000 for S44 in the 7th week and 5,300,000 for 20y28 in the 4th week, with the exception of the jamb 3Y1 which highlighted the counts higher at 5°C with 3,000,000 in the 3rd week.

The strain which showed increased longevity was 20y28 that, at the 74th week of observation, yet permitted count equal to 600,000 cells/ml. The strains less resistant in relation to the type of water, but regardless of the temperature, were: in water 1, the strain 3Y1 which first has to be reduced to the value of the *inoculum* and after extinct and, in the water 2, S44 which first lost vitality.

While the variable “temperature” has influenced as expected both the growth and the survival of the strains, the influence of the water type has led to situations more difficult to interpret. In fact, because of the higher concentration of inorganic elements and organic nutrients in water 2, it seemed reasonable to expect higher growth and better survival compared to sample 1. Indeed, the assumption has been confirmed only for what regards the survival: Figures 1 and 2 show that, although not so striking and with some exceptions, in water 2 the period of extinction of the strains - apart from the incubation at 5°C - focused between the 23rd and 53rd week with an increase of a few weeks compared to water 1.

Concerning instead the achievement of maximum cell concentration, the observed results have shown, for all strains, a substantial lack of influence of the type of water, except for the incubation at 25°C, temperature at which the water 1 appears to represent a *habitat* more favorable.

Finally, the adaptability of the jambs to the substrate “water” and its various chemical and physical conditions does not appear to be influenced by the origin of the strains, of which it is recalled that two (S44 and A) were isolated from animals and two (20y28 and 3Y1) from the water environment. Indeed, only two of the four strains, S44 and 3Y1, showed a significant adaptation to one of the two samples of water: the survival of S44 in water 1 is increased of 5 weeks at 25°C, of 16 at 15°C and of 38 at 5°C, while, in the case of 3Y1, in water 2, the survival was prolonged, compared to water 1, of 17 weeks at 25°C, of 21 to 15°C and of 26 at 5°C.

DISCUSSION

The results show that the surface water permit an active replication of *Y. enterocolitica* and also needed for their long-term survival; this observation leads us to consider more plausible the risk that the aquatic environment can be a natural reservoir of considerable epidemiological significance.

The remarkable adaptability of the organism to the aquatic environment was already highlighted in a comparative study (19) in which emerged that *Y. enterocolitica* could fit better than *Escherichia coli* and *Campylobacter jejuni*, suggesting the implementation of complex survival strategies (20, 21).

The finding of higher bacterial *inoculum* even after 74 weeks of incubation at 5°C is supported indirectly by the results of other few studies in which to our knowledge has been checked long-term survival: van Oye (1978) (22) reports that they found the presence of viable cells even after 900 days in pond water filtered, Karapinar and Gönül (1991) (23) after 64 weeks in water of sterile source and with quantitative levels equal to *inoculum*, and Liao and Shollenberger (2003) (17) for at least 5 years.

Our investigations did not reveal, as mentioned, a significant influence of the organic matter content of the water in relation to both the potential replicative of the strains and to their longevity; what is observed is in agreement with the results reported by other authors (9), that in distilled water, they observed an increase in the number in the first 2-3 days and still higher values to *inoculum* after 10 days of incubation, and in contrast with the results of van Oye (1978) (22) showing a survival limited to 45 days in samples of water low content in organic matter and lasted to 900 days in samples rich.

Regarding the role of the incubation temperature, the collected data were partially expected (19, 23) and confirm that the most suitable survival temperatures are next to 5-6°C. The higher bacterial count, however, has been ascertained at 15°C, in apparent contrast with what was observed by Highsmith *et al.* (1977) (9), who, not having taken into account a temperature value next to that common environmental temperature at our latitudes, refer to bacterial counts more elevated at 25°C than at 4°C and 37°C.

Finally, with respect to the variable represented by the origin of the strains, the findings do not appear to be sufficient to draw definite conclusions, as wide variation was revealed, with the exception of the S44 strain (swine-origin), that showed

in the water 2 - for all incubation temperature - a longevity significantly lesser than the two strains “environmental” and the other strain derived from pigs.

Although the collected results reveal an overall long-term persistence of *Y. enterocolitica* in the aquatic environment, it must temper the findings in the laboratory with broader ecological and environmental considerations because it is clear that in nature many variables are involved, first of all the competition exerted by protozoan predators (24, 25) to modulate microbial growth.

In conclusion, surface waters may be an important reservoir for the biotypes considered and thus, they may play a role in the epidemiology of *Yersinia* infections.

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